IPVS2022

26th international pig veterinary society
congress – rio de janeiro – brazil

June
21st–24th

Proceedings
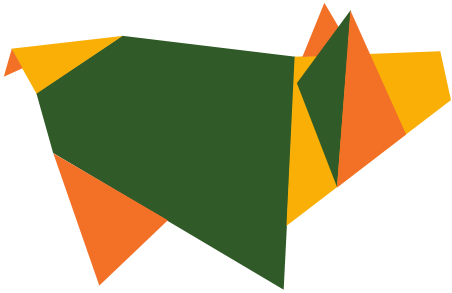# IPVS2022

Final Revision - Volume I

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RIO DE JANEIRO
2022

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IPVS2022: Memories of resilience

It had all started in Dublin (June 2016), when Brazil got the right to organize the most important congress of the international pig veterinary community: the IPVS Congress. It was a great honor, but also a tremendous responsibility. As so, we needed to start immediately with the conference planning, in other words, bringing our dreams into the real world. It took around two years with the planning, and after the IPVS2018 China, the work got even harder each day: sponsorship contacts, putting together attractive scientific and social programs.

As the Chair of the IPVS2022 Congress, I should say that our event will be known as the congress of resilience: we overcome African Swine Fever by creating a Biossecurity committee, performing a nice risk assessment, and moving the host city to Rio de Janeiro. By then, we had to rearrange our plans to adapt for the new conference venue and establish a whole new structure within a few months. And we made it! Our congress was ready to go and beautifully organized to take place from June 2nd to 5th. However, unexpectedly, the whole world faced the COVID19 pandemic in the beginning of 2020. Given this scenario, the IPVS Brazil Local Organizing Committee, supported by the IPVS Board and the sponsors, decided to postpone our event to 2022. It was a tough, but a wise decision, aiming for the safety of all involved in our event (delegates, sponsors, and organizers).

After all that has been said, here we are to accomplish this great achievement: the IPVS2022! In this sense, we are pleased to deliver to the international pig veterinary community over 450 abstracts and almost 200 lectures, which are compiled in the IPVS2022 Proceedings. We wish to thank the Scientific Committee members for putting together this sensational scientific program, the speakers for sharing their knowledge and the reviewers for reading, scoring the abstracts, and assisting with the organization of the program. We would also like to thank the IPVS Board members, all sponsors for their support, which was crucial to preserve the LOC's soundness, and the suppliers. Therefore, our dream came true: Welcome to the IPVS2022! It is a great honor to have you all with us in Rio de Janeiro or attending online. Thank you very much for being here with us, and hope that you enjoy our conference, which was kindly prepared to meet the world's pig industry's demands.

Prof. Fernanda Almeida
IPVS2022 Chair

Preface

As mentioned by our president, "Resilience" or "Persistence" would represent very well the IPVS2022 by all aspects stated by her, but also considering the attempts to bring this great event to Brazil for the second time.

For this IPVS2022 the organizing committee tried to innovate and decided to offer six options of pre-conference sessions: Antimicrobials, Agrobusiness, African Swine Fever, Mucosa Immune Response and Vaccinology, Nutrition and Reproduction, that will be held in the morning and afternoon of the opening day. At the Opening Ceremony, we will have the Tom Alexander Memorial Lecture, by Dr. Robert Friendship, talking about "Simple things – the basic principles of swine health management". For the congress, we have chosen 12 subjects divided in 20 sessions and each of these sessions will have a keynote speaker to introduce the major topics. The final program consists of 122 oral presentations, chosen among more than 480 abstract submissions. This number of abstracts imposed a great task for the reviewers from all parts of the world. For all their support, we wish to thank the following reviewers for reading and scoring the abstracts:

Abelardo Silva Jr
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For the convenience of the readers the proceedings is consistent of only one volume organized in sections comprised by the I) Tom Alexander lecture, II) manuscripts from the speaker from the three pre-conference sessions, III) manuscripts from the keynote speakers from all sessions, and IV) all abstracts also divided by thematic area.

The Scientific Committee hope that the information contained in this book can be disseminated and shared to all interested professionals in up to date scientific results that certainly will contribute to the advancement of knowledge in different areas of swine production!

Prof Roberto Guedes
Chair of the Scientific
Committee

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IPVS HISTORY

The specialization in Veterinary Medicine and the current increase in the importance of swine production have demanded the cooperation of Veterinary professionals from all over the world. This cooperation aimed the development of know–how to solve problems related to raising and reproducing this animal species.

The IPVS – International Pig Veterinary Society – was founded with this objective and with the purpose to promote, every two years, a meeting with professionals from the swine production chain to discuss the studies developed by the international scientific community.

In the last 50 years, this IPVS objective and purpose have been achieved. Since the first conference held in Cambridge/England, in 1969, the scientific and technical community had shown its interest in this international forum, where all the problems related to pig production were presented and debated. Thanks to the dedication and seriousness with which IPVS representatives have treated the issues of this community´s interest, more than 40.000 people have already had the opportunity to participate in IPVS conferences, with the presentation of more than 13.000 scientific papers. All the past editions of IPVS congresses are cited below.

HISTORICAL DATA

The interest in technical–scientific development is the main motivation to organize IPVS Congresses. Many of the debates have led to the development of procedures, which were incorporated into pig production systems, aiming to increase productivity. At each Conference, a complete review of the new advances is presented in search of more efficient solutions to face pig production challenges.

Edition	Local	Date	Chair	Abstracts	Participants
1°	Cambridge, England	23 – 28/06/69	Dr. PD Storie–Pugh	123	500
2°	Hannover, Germany	23 – 26/05/72	Dr. W Schulze	179	900
3°	Lyon, France	12 – 14/06/74	Dr. J Tournut	187	854
4°	Ames, US	22 – 26/06/76	Dr. W Brandt	374	1250
5°	Zagreb, Yugoslavia	13 – 15/06/78	Dr. O Bohm	181	450
7°	Mexico City, Mexico	26 – 31/07/82	Dr. RR Necoechea	360	1250
8°	Ghent, Belgium	27 – 31/06/84	Dr. MB Pensaert	187	854
9°	Barcelona, Spain	15 – 18/07/86	Dr. JL Garcia–Ferrero	455	1026
10°	Rio de Janeiro, Brazil	14 – 17/08/88	Dr. L Roppa	368	1233
11°	Lausanne, Switzerland	01 – 05/07/90	Dr. H Keller	528	1718
12°	The Hague, the Netherlands	17 – 20/08/92	Dr. J Verheidjen	696	2004
13°	Bangkok, Thailand	26 – 30/06/94	Dr. S Laungtongkum	538	1621
14°	Bologna, Italy	07 – 10/07/96	Dr. E Seren	648	1614
15°	Birmingham, England	05 – 09/07/98	Dr. C Glossop	839	1800
16°	Melbourne, Australia	17 – 20/09/00	Dr. R Cutler	605	1614
17°	Ames, US	02 – 05/06/02	Dr. H Harris	688	1500
18°	Hamburg, Germany	27 – 01/07/04	Dr. H Bossow	872	2455
19°	Copenhagen, Denmark	16 – 19/07/06	Dr. B Nielsen	934	2486
20°	Durban, South Africa	22 – 26/26/08	Dr. DPB Evans	918	1900
21°	Vancouver, Canada	18 – 21/07/10	Dr. E Sanford	1149	2716
22°	Jeju, South Korea	10 – 13/06/12	Dr. WH Lee	–	3099
23°	Cancun, Mexico	08 – 11/06/14	Dr. A Stephano	978	2560
24°	Dublin, Ireland	07 – 10/06/16	Dr. P Kirwan	1100	3552
25°	Chongqing, China	11 – 14/06/18	Dr. Y Hanchun	903	5599

Summary

PRE-CONGRESS – AFRICAN SWINE FEVER

CONGRESS – VIRAL DISEASES

CONGRESS – BACTERIAL DISEASES

CONGRESS – IMMUNOLOGY & VACCINOLOGY

Simple Things – the basic principles of swine health management

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Abstract

There are some simple concepts that have greatly helped to advance swine health and production. In hindsight, many important ideas appear to be obvious solutions but often it has taken wise men and women to point them out or champion these "simple things" that have helped the swine industry make amazing changes over the past 50 years. In this paper, I have chosen 5 basic concepts that have greatly improved our understanding of swine health management. Firstly, the creation of high health herds resulted in a need for the development of practical and effective biosecurity such as the unidirectional flow of pigs in a genetics company from the nucleus to the multipliers to commercial farms. Secondly, recognition that stopping the recycling of disease in the nursery is a key part of eradicating a disease from a swine operation. The development of segregated weaning and multi-site production has helped to stop or reduce the infection of naïve piglets after weaning and reduce the chance of the nursery acting as a reservoir of disease. Another simple concept that is important in swine health management is the realization that most endemic swine diseases have multiple contributing factors and that an infectious agent may be only one component and that clinical disease requires additional factors including nutrition, management, the environment etc. Disease outbreaks can occur when one small change in these contributing factors creates a "tipping point". A fourth "simple thing" is understanding that the approach to controlling endemic disease on a pig farm generally relies on a combination of maximizing the immunity of the group of pigs and minimizing the challenge of the pathogen. Often, either a vaccination program or a sanitation program is not enough alone, a combination is required for best results. And finally, the fifth thing that has greatly contributed to our understanding and our ability to control disease on farms is the use of numbers, especially production records, to help identify problems and then used to decide on a treatment plan and measure the success of the intervention. Swine health management has developed a population medicine approach and as data become easier to collect and analyze, this will increase in importance.

Introduction

I'm very honoured to have been asked to present the Tom Alexander Memorial Lecture. This is particularly meaningful for me because, as a member of the organizing committee of the 21st IPVS congress in Vancouver, I helped to establish the first Tom Alexander Memorial Lecture. In addition, I do have several connections to Dr. Alexander. For example, when I started my faculty career at the Ontario Veterinary College (OVC), part of my responsibilities involved working in the Specific Pathogen Free (SPF) repopulation centre that Dr. Alexander helped establish in the late 1950's during his time as a graduate student at OVC.

One of the reasons we had for creating the Alexander Lecture was to honour the memory of Professor Alexander who was instrumental in establishing the IPVS and supporting the organization over many years, including attending every meeting from the first one in Cambridge in 1969 until Hamburg in 2006. In addition, we intended for the lecture to be reflective, and be a chance to remember the contributions of not only Dr. Alexander but of the many scientists and veterinary practitioners who have helped the swine industry grow and improve world-wide. It is truly amazing the changes that have occurred in pork production from 1969 (when the initial meeting was held) until today, and it is important that we take time to look back at this remarkable achievement. We all owe a debt to the many innovators and great thinkers in the swine veterinary world and the educators and mentors like Professor Alexander. The saying, "you can see a long way, if you are standing on the shoulders of giants" is very appropriate in the field of swine health management.

The swine industry, worldwide, has embraced change and seen the introduction of amazing new technologies. These changes have resulted in increased productivity, food safety and animal welfare. However, it is also interesting to reflect on the very significant challenges the swine industry has faced and overcome (for the most part) in the past 50 years. In many cases, the challenges have been the impetus for changes and improvements. It could be seen as a blessing that we work in a field that seems to have an unlimited supply of challenges because with challenges come

opportunities. In the swine veterinary world, there has been a lot of good luck, particularly if you define luck as the combination of preparation and opportunity. The IPVS congress has been a source of that preparation, creating a forum for the exchange of ideas from around the world, encompassing a wide range of topics and encouraging the interaction of a wide spectrum of specialties.

In preparation of this lecture I tried to keep in mind that the audience represents swine health workers from all over the world and from multiple disciplines, so I realized the topic of the presentation needs to be broad in scope and should tie into the theme of this year's IPVS congress which is "New perspectives for swine production: biosecurity, productivity and innovation". I have reviewed the previous 4 Tom Alexander Memorial Lectures. In the first lecture in 2010, Dr. Hank Harris reviewed Dr. Alexander's many accomplishments and I encourage everyone to read these proceedings for more insight into Dr. Alexander's remarkable life and his contributions to veterinary science (Harris, 2010). The next lectures were presented by Drs. Dan Tucker, John Harding, Jill Thomson, Trevor Drew and Peter Davies, in 2012, 2014, 2016, 2018 and 2020, respectively. I have noticed that each speaker in their own way includes a vision of the future, which is always a brave (or possibly fool-hardy) thing to do in the swine world. Of course, as an opening presentation there is a need to be positive and if possible inspirational. For example, Dr. Harris predicted by 2020 that the porcine reproductive and respiratory syndrome (PRRS) virus would be eradicated from the major swine producing regions. Sadly, Dr. Harris missed the mark with his prediction, but I think his prediction was in the proper spirit of a keynote talk. To quote Elon Musk, "I would rather be an optimist and be wrong, than a pessimist and be right".

To summarize my thinking in determining the subject matter for this presentation, I have tried to be reflective, and optimistic and to create a talk that will appeal to a broad audience. While I grappled with deciding on an overall theme, I read a quote from the famous scientist D'Arcy Thompson who wrote, "It behooves us always to remember that in physics it has taken great men to discover simple things" (Thompson, 1917). I think that is true in veterinary medicine as well as physics. There have been many solutions to swine health problems, that once they have been described, seem simple and obvious. In all areas of science there is often a lag between the emergence of a problem and the eventual discovery of a solution, even when the solution in hindsight seems obvious. For example, the can opener was invented 50 years after the can came into common use. The simple things that Dr. Thompson was referring to in physics were things like an explanation for apples falling from trees or the ripples on a pond. In swine health management, simple things include how diseases continue to recycle within a population of pigs, and how the same pathogens can be present on all farms but clinical disease only occurs on a few farms.

I have chosen 5 "simple things" or more precisely, ideas that have greatly improved our understanding of swine health management. The general categories of these ideas are: practical biosecurity, disease eradication by controlling nursery pig flow, understanding that most endemic pig diseases have multiple contributing factors, endemic diseases are controlled by maximizing immunity and minimizing challenge, and the application of production records to solve health problems.

Biosecurity-getting the balance right

There was little thought paid to biosecurity, at least at the level of the individual farm, until it became possible to create "high health" herds that were free of the common endemic pig diseases. The idea of performing a Caesarian section on sows to create specific pathogen free (SPF) pigs to repopulate or establish new herds originated in the mid-western United States with the first SPF repopulation site established in 1956. Dr. Alexander, as a post-graduate student at the Ontario Veterinary College, helped establish an SPF centre in Guelph in the late 1950's. After he returned to the U.K. as a faculty member at Cambridge, he was approached by Ken Woolley (founder of Pig Improvement Company (now PIC)) to provide health advice to the newly established genetics company. He advised establishing the genetic nucleus herd by SPF means, and went further to create the breeding pyramid where animals move from the nucleus herd to the multiplier herds to the commercial farms with a one-way flow. The main principle was that animals never return from a commercial farm to the multiplier or from the multiplier to the nucleus herd. Dr. Alexander described this application of SPF and the flow of pigs in his presentation at the IPVS meeting in 1969 (Alexander & Woolley, 1969). This structure of pig flow is commonplace today and it has been adopted by almost all companies selling breeding stock. What was remarkable about the application of practical biosecurity principles with SPF technology is that many others had failed to get the balance right. If biosecurity is too extreme and rigid, then farm operations become restrictive and expensive, but if not enough attention is paid to biosecurity, then herds break with disease too readily.

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In the decade after the establishment of the first centre to create SPF pigs, there were over 60 facilities established in North America but by 1969 almost all had closed, the ones remaining being used mostly for creating research pigs or introducing new genetic lines to established herds. This was at a time when SPF was the only possible means of ensuring a herd was free of swine dysentery, atrophic rhinitis, enzootic pneumonia, and mange. These diseases were very hard to control, so there was tremendous advantage of having an SPF herd, but in general the SPF technology was not accepted or used to its full advantage. High health herds frequently broke with disease and the consequences of disease breaks were costly. The simple thing that Dr. Alexander added to the SPF technology was a practical measure- the unidirectional flow of pigs from the nucleus and the beginning of a workable biosecurity program that provided a fairly high level of protection from disease entry but still was not too restrictive to make normal farm operations impractical. If we use the analogy of the can and the can-opener, the high health herds created by SPF programs were like the cans, and practical biosecurity programs were like the can-opener.

I think we can agree that on-farm biosecurity is still a work in progress, and that we haven't perfected the can-opener. Every new disease threat over the past 50 years has resulted in advances in biosecurity. This is a great example of challenges creating opportunities. Porcine reproductive and respiratory syndrome has stimulated a large number of advances, including consideration of filters for air inlets and porcine epidemic diarrhea and African swine fever (ASF) have stimulated renewed interest in feed as a possible means of disease entry. When faced with the high cost of a disease entering a herd, it is hard not to over-emphasize biosecurity at the cost of management flexibility, it's a difficult balance.

The nursery can be a disease reservoir

Dr. Alexander also contributed to our understanding of another "simple thing"; that to eradicate disease from a herd, you need to prevent weaned piglets from becoming infected. This idea has led to segregated early weaning and multi-site production (Harris, 2010). Dr. Alexander used the technique of removing pigs from sows at an early age and raising them separately and medicating them to restock a nucleus herd with pigs free of *M. hyopneumoniae*. The idea of raising newly weaned pigs on a separate site from the sow herd has been widely used around the world. The emphasis on nursery pig health has been a key component of eradicating diseases such as porcine reproductive and respiratory syndrome virus (PRRSv) and porcine epidemic diarrhea virus (PEDv). The basic principle that Dr. Alexander described; utilizing the passive immunity of the sow to protect piglets until weaning, creates a pig that is negative for the disease agent such as PRRSv, but as the passive immunity wears off, the weaned pig is susceptible if exposed to the disease. The nursery is typically the stage in production where disease cycles within the herd, where naïve animals contact disease carriers and then once infected, they act as sources of disease for the next group of weaned pigs. Nursery depopulation is commonly used to break this cycle if simple all/in-all/out flow of the nursery rooms is not sufficient. The nursery is a hot spot for many diseases. There are many papers written about the stress of weaning, listing all the factors such as feed change, mixing with other pigs leading to fighting and the stress of transportation etc. In my opinion, not enough emphasis is placed on the fact that this is the time in the pig's life that passive immunity wanes and the pig is challenged with all the endemic pathogens present on the farm. The swine nursery is the equivalent to the daycare or kindergarten situation faced by children. All parents of young children are familiar with the unending problems of runny noses and respiratory symptoms of kindergarten children, and not very long ago this list would have included measles, mumps and chicken pox that all children contracted as they started school. They develop these diseases not because of diet change or stress from their environment but because they are exposed to disease organisms and unlike adults, they have no or little immunity. To eradicate a disease from a farm, it is important that the nursery doesn't remain a reservoir of active infection.

The tipping point

On the other hand, many pig diseases can't be eradicated, and generally we have developed strategies to keep them under control. The fact that the pathogens are present on almost every farm but only certain farms have outbreaks of clinical disease is sometimes difficult to understand. The reason for the sporadic nature of clinical signs is because these diseases are usually a result of multiple contributing factors, with the pathogen being only a piece of a large puzzle. Morris et al. (2002) have described the multifactorial causation of most endemic pig diseases as follows; "Most of the diseases we deal with in intensive production systems have multiple component causes. Some of these may be necessary causes, in that the disease cannot occur without them being present, but they are not sufficient causes when acting alone. Koch's postulates therefore have only limited relevance to current examinations of disease causation. Diseases for which presence of the agent alone is both necessary and sufficient cause for expression of disease are

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usually the easiest to control, so we have eradicated most of these diseases from intensive units, leaving only those with more complex causal webs as our current challenge. One reason why veterinarians become confused by apparently conflicting research and field data about disease causation is that there are often multiple component causes of disease expression, and various mixes of these may create sufficient cause, thus making it seem that disease occurrence is capricious, and also producing apparently inconsistent results between studies, simply because different component causes varied in the different investigations."

The popular author Malcolm Gladwell in a book entitled "The Tipping Point -How Little Things Can Make a Big Difference" describes how the human brain tends to think in a linear manner, expecting a small change to produce a small consequence, but there are lots of examples particularly in the case of disease outbreaks where a small change on a pig farm can suddenly trigger something quite big, namely disease outbreak (Gladwell, 2000). Dr. Tim Blackwell presented this concept at an American Association of Swine Veterinarians annual meeting to explain disease outbreaks on pig farms (Blackwell, 2001). For example, in the case of post-weaning diarrhea, a farm may have numerous risk factors such as poor cleaning practices, very young weaning age, a starter diet with a high proportion of plant protein, but the pigs may remain healthy until a cold night causes the pigs to be chilled and that is the tipping point that triggers a severe outbreak of *E. coli* diarrhea. A similar outbreak might occur on another farm but the factor that triggered the disease or tipped the scales might be something completely different, such as a change in cleaning procedure. An additional reason that these multifactorial diseases are difficult to understand is that even if the triggering factor can be identified, the disease can't be easily controlled simply by fixing the factor that triggered the disease because after the outbreak the situation has changed. In this case, warming the pigs will not be enough, and it may require addressing several of the other contributing factors before the situation is stabilized. Too often in the past an outbreak of post-weaning diarrhea would result in medication with an antibiotic directed at *E. coli*, but no further investigation of the contributing factors would occur.

Swine disease research is generally performed in controlled research facilities focussing on a single pathogen in order to understand the infection mechanism and the animal's biological response. There are a few studies which have tried to measure the interaction and impact of two or more pathogens infecting pigs either in a controlled facility or even occasionally on-farm in a natural outbreak. For example, several studies have shown the synergistic effects when pathogens such as *M. hyopneumoniae* and PRRSv co-infect a pig. These studies are complicated and difficult to replicate when they are moved from the laboratory to the farm. There are a few studies that have attempted to work with on-farm conditions and measure the impact of a high health challenge compared to a lower health challenge (e.g. Cornelison et al., 2018). Generally, these studies attempt to classify a high or low challenge based on pathogens present and not the multiple contributing factors but these studies are a good start in increasing our understanding of multifactorial diseases. To study the interaction of management-environment and host factors, in addition to the interaction of several pathogens and even to evaluate the role of the animal's microbiome and genetic make-up is an infinitely complex task. Clearly we have a long way to go to completely understand and control multifactorial diseases.

Maximizing immunity/Minimizing the challenge

In general, the current approach to controlling an endemic multifactorial disease relies on a combination of two basic strategies; firstly, to ensure that pigs maintain a high level of immunity against the disease, and secondly, to ensure that the pigs are exposed to the smallest possible pathogen challenge. This seems very obvious but it is sometimes useful to keep this simple plan in mind when efforts to control a disease are not working. There are many examples of pig diseases that can't be controlled by available vaccines alone, but with the addition of increased hygiene or reduced stocking density or better ventilation, the clinical signs disappear. Over the past few decades, there are good examples of disease problems being controlled to a great extent with this strategy. For example, enzootic pneumonia is much less of a problem today because of the combination of vaccination at the nursery stage combined with all-in/all-out management and good ventilation in the grower-finisher barn.There are various reasons for a pig to have lower than ideal immunity including insufficient colostrum intake, nutritional deficiency, or possibly chronic stress, in addition to lack of previous exposure to a pathogen that would have triggered antibody production. Some diseases are highly contagious and impossible to control through hygiene or even all-in/all-out pig flow and so control relies on ensuring widespread immunity. The control of porcine circovirus through vaccination is a good example of this situation. Until vaccines were introduced, attempts to control porcine circovirus were universally ineffective. Whereas, there are examples of diseases where attempts at boosting immunity using vaccines have not been successful but methods to minimize the challenge through improved hygiene, slatted flooring and all-in/all-out pig flow have worked well. Diseases such as pleuropneumonia caused by *Actinobacillus pleuropneumoniae*, and swine dysentery have proven difficult to control with vaccination but virtually disappeared (at least in Canada) with the widespread use of

all-in/all-out management of grower-finisher barns and other management strategies to reduce the pathogen challenge. Likewise, housing and sanitation have controlled internal parasites and coccidiosis to a great extent.

Numbers – the value of production records

Just as the widespread use of cans necessitated the invention of the can-opener, the rapid increase in herd size which occurred in North America in the 1980's, necessitated much greater emphasis on record keeping to keep track of the daily tasks in a pig operation and this fortuitously coincided with the arrival of personal computers and the development of software packages that enabled veterinarians to evaluate herd data to solve problems. Production records were almost non-existent when I first started visiting swine farms, but for the past 40+ years, the analysis of records have been an integral part of swine practice. The common saying *"if you can't measure it, you can't improve it"* (generally attributed to Peter Drucker, the author of many books on business) applies to pork production. The extent to which record analysis can be used in problem-solving reproduction issues is well summarized by Dr. Koketsu in a keynote address at the 2016 IPVS (Koketsu, 2016). Using records as a tool in veterinary practice has led to an appreciation of the significant cost of endemic diseases that in the past had been referred to as subclinical diseases, which implied they didn't produce an effect on the pig, but careful monitoring using production records have shown these subclinical diseases can be economically important diseases. In addition to identifying production problems, records are often essential in deciding the best course of action in rectifying the situation (e.g. creating various scenarios using partial budgeting or other techniques to predict economic impact of the intervention) and then in measuring the outcome to ensure the corrective measure was effective. The use of production records transformed the approach veterinarians used to tackle health problems in swine to a population medicine approach (Polson et al., 1998) As every aspect of intensive farming becomes more mechanized and electronics become even more widespread, the amount of data is greatly increasing and the ability of farm managers and veterinarians to identify problems quickly and to measure outcomes precisely will continue to improve.

Conclusions

The swine industry throughout the world has made great advances in the past 50 years. There have been many remarkable scientific discoveries which have contributed to the increases in production efficiencies, food safety, animal welfare and decreased environmental impact, but there are some "simple things" that have helped guide the amazing progress that has been made in swine health management. We owe a great deal of gratitude to people, like Dr. Tom Alexander, working in the swine health field, who have through their leadership and mentoring, helped to increase our understanding and our recognition of these basic concepts.

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What's in your cooler? Understanding semen dose quality variation

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Introduction

Two decades ago, the basic description of what constituted a semen dose was broadly accepted as approximately 3 to 4 billion sperm, in doses of at least 80 ml (Althouse & Kuster, 2000; Singleton, 2001). Although there were differing perspectives on whether sperm targets should be based on total sperm or adjusted based on perceived "viability" during processing, and on how much weight to give various quality parameters when deciding whether a particular ejaculate should be processed (Althouse & Kuster, 2000; Singleton, 2001). Around this time, adoption of post-cervical AI (PCAI) technology was ramping up in some parts of the world (Garcia-Vazquez et al, 2019). However, as recently as 10 years ago, traditional intracervical (CAI) semen doses as described above were still the norm in North America, with (PCAI) beginning to be introduced as a viable option at the commercial level, along with lower sperm numbers typically contained in the smaller volume PCAI doses (Kuster, 2011). The goal of this presentation is to provide objective descriptions of current semen doses from around the globe, as well as examples of variation within and between boar studs in the same region, and brief context on how laboratory analysis methods can affect perceived dose quality.

Background

Since the beginning of swine AI in the 1930's, a multitude of trials have been conducted attempting to identify the optimal semen dose, and extensive experience has been gained in mature swine industries, which has produced a diverse set of outcomes which will be reviewed in the presentation (Polge, 1956, Waberski et al, 2019). Asymptotic models have been proposed to describe the relationship between the number of sperm inseminated and the probability of conception (Schwartz et al, 1981). The pregnancy rate and litter size initially improve with increasing number of spermatozoa in the insemination dose, however, at a higher level, these variables reach a maximum at which point the fertilization of all oocytes is no longer limited by sperm number (Woelders, 1991). Once the asymptote of the fertility curve is reached for a particular ejaculate or semen pool, no further improvement is expected simply through increasing sperm number alone (Flowers, 2008). However, survival rates of the zygotes and embryos after fertilization can still be affected by non-compensable sperm factors unresponsive to the number of sperm in the dose (Evenson, 1999; Pinton et al, 2000).

Materials and Methods

The data presented below were obtained in 2021 with the explicit goal of compiling in one resource accurate descriptions of what constitutes a semen dose from representative swine production regions around the globe. Actual dose analysis results were preferentially sought to quantify what is actually in production doses, in contrast to what is supposed to be in the doses. Individuals and institutions with direct access to such information were asked to provide common parameters in a standard format. Information was generously returned by a number of knowledgeable sources from across the global swine industry. Please refer to the Acknowledgments section for affiliations by region.

The tables below are arranged in three distinct categories: quality assurance (QA), quality control (QC) and Targets/Minimum Standards. Quality Assurance (QA) data was reported from laboratories external to the site(s) of production. Data from QA laboratories is generally regarded as the most reliable, as it involves a third party measuring what is actually in the dose. However, since QA metrics are lab-specific, differences in analysis equipment and procedures may vary between locations, and these potential differences must be considered when comparing outcome measures. The QA metrics are presented as means and standard deviations of all routine doses submitted to the respective laboratories. These aggregate numbers are not meant to represent any particular boar stud, many of which have unique site-specific targets and characteristic variation. Quality Control (QC) data was provided by boar studs, representing internal analysis of their own production, or shared between production sites. Compared to QA results, QC data is typically more narrowly focused within a particular location or system. The summary table on industry targets are general industry expectations, boar stud goals, genetic company guidelines, or production company metrics that are aspirational, meaning actual results may diverge from intended standards. Unless otherwise noted, all values presented below are from calendar year 2020.

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Results

Quality assurance values were available from three laboratories s in the USA, two in Spain and one each for Canada, Mexico, Germany and Australia.

Table 1. North America (USA) - Intracervical AI doses (CAI)

		Volume (ml)	Concentration x10^6	Total Sperm x10^9	Motility %	Normal Morphology %
USA[1]	Mean	71.6	46.7	3.34	82.9	80.8^
	SD	3.2	5.5	0.41	5.2	6.3
USA[2]	Mean	71.4	42.9	3.05	84.1	77.9^
	SD	3.9	6.4	0.45	6.1	7.3
USA[3]	Mean	75.7	42.0	3.16	75.5	86.5*
	SD	3.2	6.4	0.48	13.5	5.2

^ USA[1] & USA[2]: morphology values = full microscopic differential; *USA[3] = CASA morphometry

Table 2. North America (USA) - PCAI doses

		Volume (ml)	Concentration x10^6	Total Sperm x10^9	Motility %	Normal Morphology %
USA[1]	Mean	40.0	48.6	1.61	86.1	76.7^
	SD	3.2	5.5	0.26	4.9	7.7
USA[2]	Mean	38.9	42.2	1.63	83.5	78.1^
	SD	6.1	5.4	0.29	3.9	5.2
USA[3]	Mean	51.2	41.8	2.13	71.0	89.1*
	SD	4.8	6.1	0.31	16.1	5.0

^ USA[1] & USA[2]: morphology values = full microscopic differential; *USA[3] = CASA morphometry

Table 3. North America (Canada and Mexico) - Intracervical AI doses (CAI)

		Volume (ml)	Concentration x10^6	Total Sperm x10^9	Motility %	Normal Morphology %
Canada	Mean	72.0	42.3	3.03	78.9	77.9
	SD	4.0	9.6	0.66	10.5	9.1
Mexico	Mean	77.2	41.1	3.17	84.8	-
	SD	5.9	7.5	0.58	9.4	-

-   Mexico = morphology not reported

Table 4. North America (Canada and Mexico) - PCAI doses

		Volume (ml)	Concentration x10^6	Total Sperm x10^9	Motility %	Normal Morphology %
Canada	Mean	43.2	39.5	1.72	80.5	79.1
	SD	10.4	4.3	0.48	5.3	6.4
Mexico	Mean	53.0	37.2	1.97	85.5	-
	SD	4.7	5.8	0.31	7.8	-

-   Mexico = morphology not reported

Table 5. Germany, Spain and Australia – Intracervical AI doses (CAI)

		Volume (ml)	Concentration x10^6	Total Sperm x10^9	Motility %	Normal Morphology %
Germany	Mean	86.8	22.9	1.99	84.2!	87.2
	SD	3.0	4.6	0.40	7.9	9.2
Spain[1]	Mean	85.0	33.0	3.04	88.7	77.0
	SD	4.5	6.6	0.61	6.3	7.6
Spain[2]	Mean	72.5	39.1	2.77	73.9	69.6
	SD	17.7	12.4	0.62	15.8	20.7
Australia#	Mean	75.7	42.0	3.16	75.5	86.5
	SD	3.2	6.4	0.48	13.5	5.2

!Germany: Day 3 motility; Spain[2]: values combined 2018 to 2020; #Australia: values combined 2006 to 2015

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Table 6. Spain – PCAI doses

		Volume (ml)	Concentration x10^6	Total Sperm x10^9	Motility %	Normal Morphology %
Spain[1]	Mean	57.6	32.8	1.89	86.7	76.5
	SD	5.5	3.4	0.38	9.7	9.0
Spain[1]	Mean	43.2	33.9	1.46	87.5	79.6
	SD	2.5	6.3	0.28	9.7	8.0
Spain[1]	Mean	33.3	32.2	1.06	86.8	79.0
	SD	3.9	5.6	0.19	7.5	9.9
Spain[2]	Mean	39.4	42.2	1.71	74.8	62.7
	SD	6.8	9.9	0.45	14.1	21.3

Spain[1]: PCAI doses reported separately for three different target volumes of 60 ml, 45 ml, and 30 ml, respectively

Quality control data, from within or between production sites was available from Norway and The Netherlands.

Table 7. Norway and The Netherlands - Intracervical AI doses (CAI)

		Volume (ml)	Concentration x10^6	Total Sperm x10^9	Motility %	Normal Morphology %
Norway	Mean	89	23.9	2.13	93.7	89.5*
	SD	-	2.7	0.24	3.5	0.3
Netherlands	Mean	80.2	20.4	1.60	81.0	82.8*
	SD	1.1	4.0	0.30	10.3	6.8

* Norway = Fresh motility and CASA morphometry; * Netherlands: Motility and CASA morphometry at Day 5

Generally accepted industry targets or minimum standards are presented for the United Kingdom, France, Brazil, and The Netherlands, with one large production system included from Spain. For additional information on minimum standards for AI dose production from around the world, please refer to Waberski et al, 2019.

Table 8. United Kingdom, France, The Netherlands, Spain and Brazil – Industry Targets

		Volume (ml)	Total Sperm x10^9	Motility %	Normal Morphology %
UK	CAI	75 to 90	≥1.80 ±10% of target	≥70% fresh ≥60% expiry	≥70%
France	CAI	80	1.5 motile	≥80% fresh	≥75%
	PCAI	75 to 80	1.5 motile		
	GEDIS	75	2.0 to 2.2	≥70% Day 3	
Netherlands	CAI	80	1.3 to 1.5 motile	-	-
				-	
Spain[3]	CAI	85	2.80	-	-
	PCAI	45	1.50	-	-
Brazil	CAI	80 to 90	2.70	≥80% CASA or	≥75%
	PCAI	40 to 45	1.40	>70% subjective	

Netherlands & Spain[3]: motility and morphology minimums not specifically reported

Discussion

Common themes that occur across most swine producing regions of the world include a drive for efficiency expressed as fewer sperm per dose while maintaining or improving sow reproductive performance. These goals have been pursued from different angles, with one notable regional divergence being reducing sperm concentration in traditional higher volume intra-cervical doses in contrast with maintaining concentration but reducing volume in doses deposited post-cervically. Examples of this include the reduction in sperm number over time from 4.0 billion in 100 ml to 1.5 billion in 80 ml in the Netherlands (Feitsma, 2009), while PCAI doses of 34 to 50 ml with 1.35 to 1.75 billion sperm are commonly measured in quality assurance doses from the USA (KRC lab data, 2020).

High standard deviations in QA laboratory data suggest a wider range of targets between the boar studs they serve. For example, the standard deviations for total sperm per CAI doses in Canada, Mexico and Spain were 0.58 to 0.66 with means of 2.77 to 3.17, indicating a broader range of targets observed in those areas compared to other regions. Averages for motility and morphology measured by the QA laboratories tended to be above the minimums described as industry targets from Table 8 and other references (Althouse 2002, Waberski et al, 2019). However, a closer look

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at the standard deviations on those same quality parameters reveal that a significant portion fell below the minimum standards, particularly for morphology. Morphology values tended to be higher for laboratories reporting Computer Automated Sperm Analysis (CASA) morphometry compared to those performing full morphology differentials microscopically. This is likely due to the fact that most CASA systems are programmed to identify fewer categories of abnormalities, as well as differences in sensitivity between rigid software algorithms compared to a competent technician at a high magnification microscope.

Between individual boar studs in the same region, differences in doses still occur, despite the generalities presented above. For example, commercial boar studs serving external customers in North America tend to observe more conservative sperm per dose targets than boars studs that are owned by production systems aiming to optimize efficiency. This is not universally the case, as German boar studs have focused heavily on quality improvement over the years, enabling them to maintain customer satisfaction with fewer sperm per dose (Riesenbeck et al, 2015). A similar pattern is present for the Netherlands and Norway. Additional variation occurs due to differing quality expectations and ability to meet those expectations based on evaluation equipment, technician training, and level of independent external review. One common source of variation between boar studs is the calculation of sperm per dose target. Some use a fixed total sperm target, while others use targets adjusted by various factors ranging from motility (total or progressive), and/or morphology (all or some abnormalities considered), to processing method (single sire or pooled), extender/expected storage period, breed/genotype, collection frequency or other unique factors(Kuster & Althouse, 2000). For this reason, when a semen supplier states a sperm per dose target, it is important to ask a follow-up question: how do you calculate the number of sperm you include in the dose?

The next consideration for dose quality is variation over time. Just because a particular boar stud has well-defined targets and historic confirmation of their quality from an independent QA laboratory, doesn't necessarily mean the doses they produce tomorrow will be the same. The sensitivity of the QA lab is instrumental in recognizing actionable variation, and frequency of monitoring determines the time lag between detection and intervention (Kuster & Althouse, 2013). Examples of commonly encountered variation over time within boar stud include technician-dependent differences in concentration results, CASA maintenance issues degrading morphology detection, and bacterial contamination. Seasonal or less frequent issues that can appear suddenly include acute issues with sperm quality due to heat stress, mycotoxins, laboratory water quality, materials toxicity, etc. (Parrish, 2017; Lyons, 2017; Althouse, 2017).

Lastly, not all laboratory results are the same. Differences in equipment, supplies, reagents, methods, and people all contribute to reported outcomes. For motility analysis, results may differ based on whether the values are subjective estimates or measured by a CASA system. If a CASA system is used, distinctions between brands and software settings become relevant. Factors independent of equipment, such as extender composition, sample preparation, and age at analysis can all contribute to differences in reported values. As suggested in the tables above, morphology results can differ when analyzed by CASA morphometry recording a sub-set of abnormal forms versus a full microscopic differential. Even full differentials hold the potential for deviation related to the type of slide preparation:stained versus wet mounts (Bamba 1987, Sprecher & Coe, 1995). Although the number of sperm classified is important, manual differentials are able to attain relevant confidence levels (Kuster et al, 2004). Similar equipment and method considerations apply to sperm concentration measurements. Calibration to a gold standard is clearly important when it comes to sperm concentration measurement, and sole reliance on a mechanical device is potentially misleading, as this effectively takes calibration out of the hands of a capable andrology laboratory director and transfers that responsibility to a manufacturer that assembled the hardware, programmed the software, and decided how well individual units would need to conform with specifications (Kuster, 2005). A concentration measurement device is just one component of a process that includes technicians sampling the dose, aliquot preparation, and often precision consumables, all of which can contribute error to the final result if not carefully managed to avoid or detect and correct such events. Culturing doses for bacteria can also lead to divergent results between laboratories based on factors such as dose age, storage temperature, type of culture media used, sample inoculation method, incubation conditions, reporting conventions (qualitative or quantitative) and lab-specific thresholds for "acceptable" types of bacteria and/or level of growth to be considered "positive" (Althouse et al, 2008; Kuster & Althouse, 2016).

Conclusion

Although various doses are successful in their own context, not all are interchangeable without considering other factors. Initial semen quality, extender composition, processing and storage conditions, semen age, bacterial content, homospermic versus heterospermic inseminations, experience of AI technicians, site of semen deposition, number of inseminations per service, and female characteristics are a partial list of factors that may interact with volume and total sperm number per dose in determining the threshold value for optimal fertility (Kuster, 2003). Definite regional tendencies are apparent in the way doses are built, but variation between and within individual boar studs can result in doses that differ dramatically from local expectations. The only sure way to know what is in your sow barn cooler

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is to have doses evaluated by a proficient andrology laboratory or to confirm the boar stud supplier demonstrates consistent satisfactory results through a comprehensive QA/QC program (Kuster & Althouse, 2013, Riesenbeck et al, 2015).

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Key Principles for Improved Sow Lifetime Performance

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Introduction

Good gilt replacement management is often overlooked as a critical driver of sow lifetime productivity (SLP). In this paper, we will review the key principles for improved SLP that affect the overall efficiency of replacement gilt production and the genetic transfer program. A gilt becomes a replacement female at birth, and individual gilt birth weight and sow birth weight phenotype are early indicators of future retention and performance. The implementation of an efficient gilt development system that induces early puberty by 200 days of age is a critical selection tool to identify gilts with the greatest potential lifetime productivity. To achieve this, direct and consistent contact with high libido boars starting from approximately 170 days of age is a critical component of a puberty stimulation program. This permits producers enough time to breed gilts at second estrus and at acceptable target weights (135-160 kg). An established Heat-No-Serve (HNS) program also permits effective pre-breeding management and allows for proper gilt acclimation and a favorable metabolic state in the pre-breeding period. This is critical as first litter size is predictive of later lifetime performance and how gilts are managed prior to first mating will have lasting effects on lifetime production. Investing in the development of the stockperson and measuring and managing the key components of gilt development are an integral part of the gilt development unit (GDU). This paper is based on earlier reviews and scientific articles presented by the authors, as cited in the references included.

Extreme (<1 kg) individual birth weight is predictive of low retention

A low birth weight is a major risk for increased pre-weaning mortality, poor growth from birth and lower retention until final selection. If selected and bred, a low birth weight limits SLP. Gilts weighing less than 1.0 kilogram at birth have increased preweaning mortality rates and have little chance of surviving until weaning (Magnabosco et al., 2015). Those gilts that do survive past the nursery phase have poor growth until finishing and are significantly lighter than their higher birth weight litter mates (Magnabosco et al., 2015). Almeida et al. (2017) reported that low birth weight affects vaginal length and ovarian follicular dynamics at 150 days of age, potentially leading to poor future reproductive performance. Gilts weighing less than 1.0 kg at birth had fewer total pigs born alive at first farrowing, fewer pigs produced over three parities and increased removal due to anestrus, compared to gilts weighing over 1.0 kg (Magnabosco et al., 2016). Non-selection of low birth weight gilts at birth, or at processing or at weaning would improve the efficiency of the gilt development unit.

A low birth weight phenotype reduces the efficiency of a gilt replacement program

A sow "low litter birth weight" phenotype at the nucleus and multiplication level carries all the same risks described above for individual low birth weight gilts but as a repeatable "litter" trait (Foxcroft & Patterson, 2020a; Patterson et al., 2020; Smit et al., 2013). This trait is repeatable over consecutive parities and arises from interactions among the component traits of ovulation rate and the dynamics of early embryonic survival, which in turn lead to excessive intrauterine crowding in early gestation and limited placental development (Da Silva et al., 2018; Foxcroft et al., 2009; Ladinig et al., 2014; Smit et al., 2013). More recently, Moroni (2020) determined that the primary driver of impaired embryonic development and lower average litter birth weight in sows with the low litter BWP is due to limited uterine capacity and lower placental efficiency leading to an unfavorable intrauterine environment at day 30 of gestation. Later in gestation, a low BWP is associated with characteristics of intrauterine growth retardation independent of the size of the litter born (Smit et al., 2013).

A low BWP negatively affects birth weight, body composition, post-natal survival and growth performance of terminal-line offspring, independent of the size of the litter born (Smit et al., 2013). In replacement females, gilts born to sows with a low BWP have poor survival to weaning and lower gilt retention rates during development (Patterson et al., 2020). Retaining sows in the production genetic nucleus population if they exhibit a repeatable low BWP negatively impacts the efficiency of replacement gilt production and represents a poor return on the investment in their high genetic merit (Foxcroft & Patterson, 2020a; Patterson et al., 2020). Nucleus sow culling strategies aimed at the early removal of the 10 to 15% of sows with the extreme low BWP, as well as non-selection at birth of the lower birth weight gilts from other litters, will improve the overall efficiency of the nucleus/multiplication farm in terms of efficient genetic transfer.

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Early responses to effective boar stimuli is a critical selection tool

An early pubertal response (a measure of sexual precocity) is strongly linked to better SLP and is one of the first indicators of future performance. Gilts with early puberty accumulate fewer non-productive days (Patterson et al., 2010), have improved retention rate and longevity (Knauer et al., 2010; Patterson et al., 2010; Roongsitthichai et al., 2013; Li et al., 2018), produce more litters and piglets during their lifetime (Tart et al., 2013) and have increased reproductive efficiency over their lifetime (Patterson et al., 2010). In a recent study, using PIC L-1050 females, gilts younger at puberty and at first mating had higher retention and total pigs born to parity 3 per gilt served (Figure 1 and 2) (Pinilla & Patterson, unpublished data, 2020). Similarly, Koketsu et al. (2020) reported that gilts with early age at first mating are more likely to become higher efficiency sows as measured by increased herd-life days and annualized piglets weaned and reduced non-productive days.

Collectively, these results suggest that "Select" gilts, those with the best potential SLP, are those that either reach puberty at less than approximately 200 days or are first bred with at least one HNS at less than 230 days (Foxcroft & Patterson, 2020b). The later maturing "Opportunity" gilts are less fertile (Foxcroft et al., 2006). Therefore, the implementation on an efficient gilt development system that includes direct and consistent contact with high libido boars from 170 days of age is a key component of a gilt selection program.

Figure 1. a) Retention rate and b) total pigs born to parity 3 for gilts served and classified according to age at puberty. From Pinilla & Patterson (2020). Different letters indicate significant difference between age at puberty classes (P < 0.05).
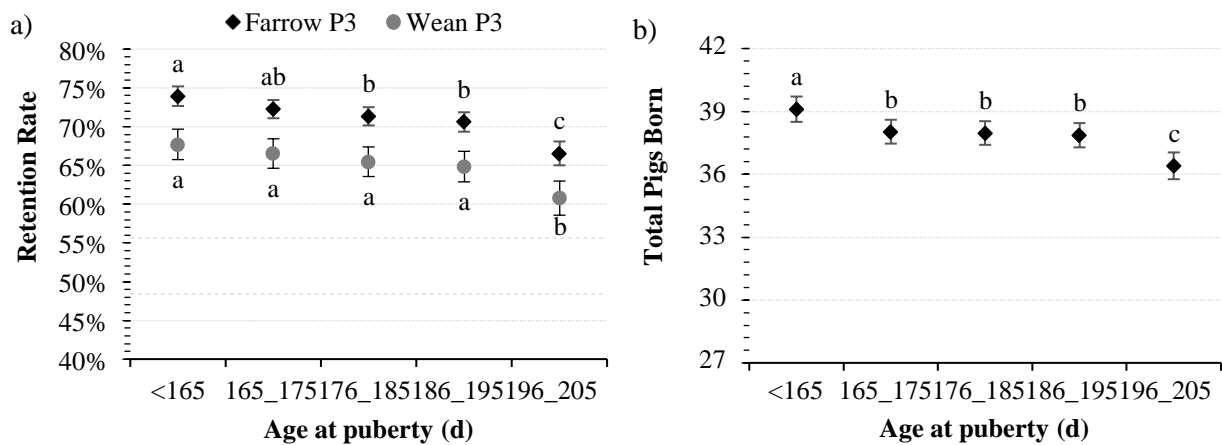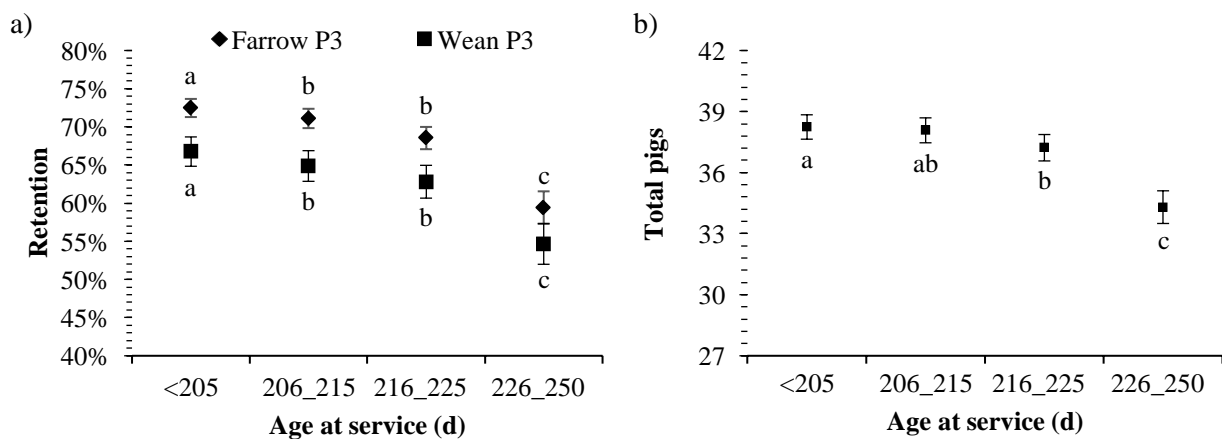

Figure 2. a) Retention rate and b) total pigs born to parity 3 for gilts served and classified according to age at service. From Pinilla & Patterson (2020). Different letters indicate significant difference between age at service classes (P < 0.05).

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Direct contact with boars is more efficient than fenceline contact in triggering puberty

Direct contact with boars is more efficient in triggering puberty and allows gilts the freedom to "solicit" the boars. The "boar effect" is the most potent stimulus for pubertal onset in gilts. It is a combination of tactile, visual, auditory and olfactory cues (Hughes et al., 1990) and daily physical exposures to a rotation of mature, high libido boars for a minimum of 10 to 15 minutes per day maximizes the response (Levis 2000; Paterson et al., 1989), per every 20-30 gilts. Direct contact with a boar reduces age at puberty and increases the percentage of gilts cycling, compared to fenceline contact (Levis 2000; Patterson et al., 2002) and taking the gilts to the boar exposure area is more effective in inducing puberty than taking the same boars to the gilts pen (Patterson et al., 2002). More recently, Knox et al. (2021) reported an earlier and more synchronous expression of pubertal estrus using direct physical boar contact compared with very effective fenceline exposure in a purpose built boar exposure area (BEAR) to boar-derived stimuli. A consistent supply of mature (older than 10 months), high libido (sexually motivated), size appropriate, mobile (an uncompromised ability to stand and walk) epididimectomized or vasectomized boars (teaser boars) is an essential, and often under recognized, component of the GDU.

Gilt behavior during the oestrus cycle can be classified as proceptive and receptive (de Jonge et al., 1994; Hemsworth, 1985; Tilbrook & Hemsworth, 1990). Typical proceptive behaviors include, searching the boar, staying near the boar and exhibiting behaviors (nosing the boar, mounting other females in the pen) to elicit a response from the boar (de Jonge et al., 1994; Hemsworth, 1985; Tilbrook & Hemsworth, 1990). When a gilt exhibits the standing reflex in the presence of a boar or in response to the back pressure test by a stockperson she exhibiting receptive behavior. In modern commercial systems, housing and the method of puberty stimulation (fenceline or in stalls) may limit gilts from displaying these natural behaviors (Hemsworth, 1985). Gilts that are housed adjacent to boars may habituate to the boar stimuli as reflected in reduced proceptive and receptive behaviors compared to gilts that are housed one meter away (Tilbrook & Hemsworth, 1990). Stockpeople may fail to realize that during the pro-estrous period it is the gilt that will actively and aggressively "solicit" the boar (Foxcroft & Patterson, 2020b). An understanding of these behaviors, the choice of puberty stimulation procedures and the stockperson involved will impact the efficiency of the gilt development unit (Hemsworth, 1985). Therefore, the key to successful puberty stimulation and estrus detection, and effective use of space and labor, is to use a properly designed Boar Exposure Area (BEAR), or at a very minimum, a system that permits direct contact with high libido boars and allows gilt the freedom to "solicit" the boars.

A recorded pubertal estrus by 200 days of age is critical

Gilts should be should be stimulated early enough (~170 days of age) to trigger early puberty and to permit producers enough time to breed gilts at second estrus and at acceptable target weights (135-160 kg). Age at puberty (a HNS event at less than 200 days) is used to determine the relative sexual maturity and reproductive potential of the gilt (Foxcroft & Patterson, 2020b). Delaying breeding to second recorded estrus has a positive effect on first litter size (Levis 2000; MacPherson et al., 1977; Walker et al., 1989; Young et al., 1990) and gilts bred at second estrus produced 1.2 more pigs after four litters compared to gilts bred at first estrus (Young et al., 1990). More recently, gilts bred at first detected estrus were reported to have lower farrowing rates and total born at parity 1 and lower lifetime efficiency as measured by retention and total pigs born per gilt served (Figure 3) (Pinilla & Patterson, unpublished data, 2020). Weight at HNS is used to manage predicted weight at breeding and given the high growth rates in commercial conditions and the industry recommendations that gilts be bred at a target weight of 135 to 160 kg, a recorded estrus by 200 days of age is critical. Therefore, implementing an efficient (30 day) puberty induction program at ~170 days is also critical for controlling the weight of gilts bred at 2nd estrus.

Heavy weights at breeding are a major risk factor for retention in the breeding herd

The medium to high growth rates of contemporary gilt on adlib feeding regimes, linked to older ages at the start of puberty stimulation and extended entry-to-service intervals, are a major cause of gilts being bred overweight (>160 kg), resulting in poor retention and early deaths. Industry recommendations state that gilts be bred at a target weight of 135 to 160 kg. The major risk factor for overweight gilts is poor retention, efficiency and longevity. It is important to focus on SLP and herd-life efficiency, not only first parity litter size, when evaluating weight at service on future performance. Gilts with increased age at first mating (Roongsitthichai et al., 2013; Schukken et al., 1994) and weight at first service (Amaral Filha et al., 2008) have been reported to have more pigs born in parity 1. However, increased service weight has been associated with a higher percentage of stillborn pigs during their first farrowing (Amaral Filha et al., 2008; Bortolozzo et al., 2009), increased removal due to lameness (Amaral Filha et al., 2010) and lower herd retention rates (i.e. lower lifetime productivity) (Amaral Filha et al., 2008; Roongsitthichai et al., 2013). The effect of heavier weight at service and lower retention was confirmed in our recent study (Pinilla & Patterson, unpublished data, 2020) (Figure 4). Similarly, although gilts with lifetime growth rates from birth to mating > 771 g/d had more total number of pigs born, they also had more stillborns pigs and more piglets born weighing less than 1.2 kg compared

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to slower growing gilts (< 700 g/d) (Amaral Filha et al., 2010). Sow longevity is also an important component of sow welfare and sustainability (Bergman et al., 2019; Engblom et al., 2007). Early removal from the herd due to lameness (Iida et al., 2020; Supakorn et al., 2018) and sow mortality (Supakorn et al., 2019) are of particular relevance to current industry trends relating to sow welfare. It is therefore, critical that breeding weight must be controlled as part of an effective gilt management program.
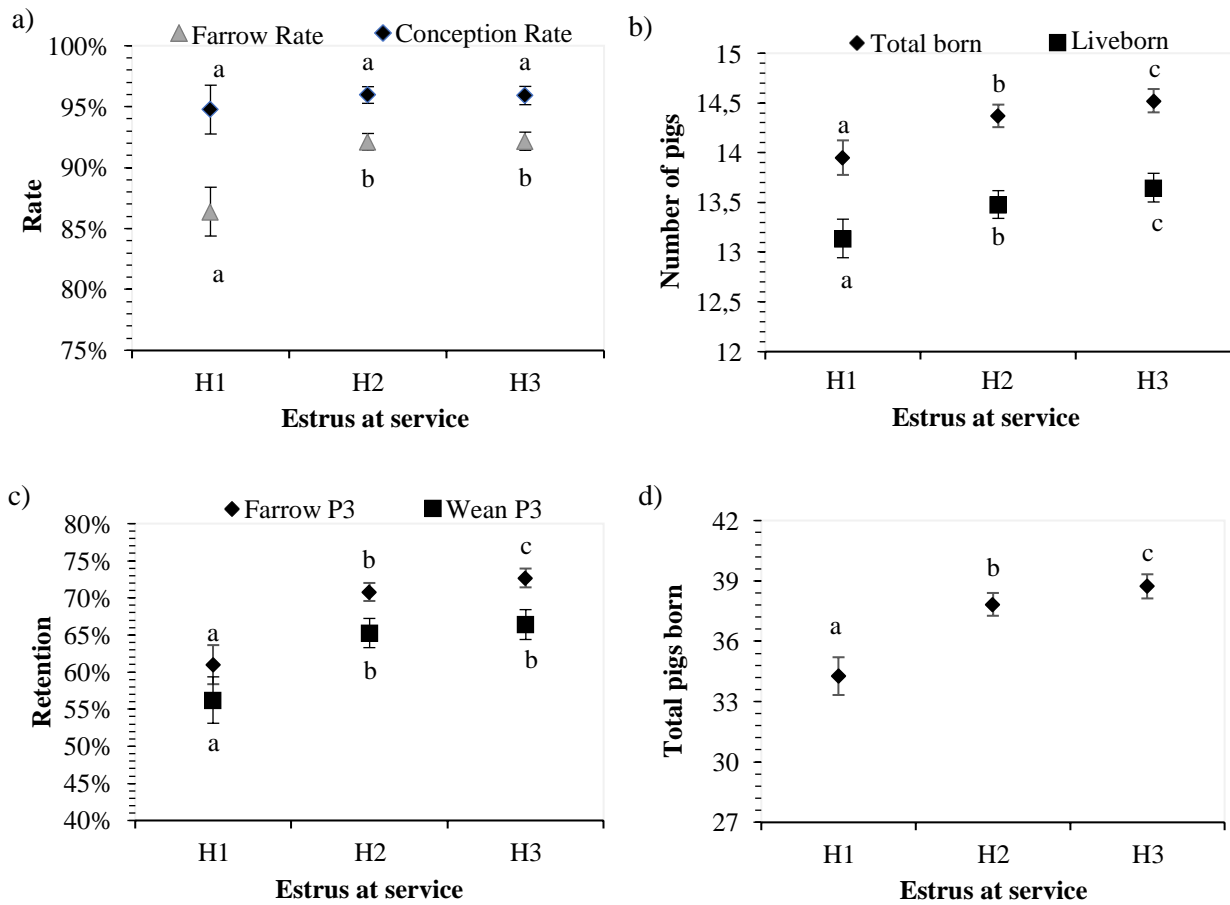
Figure 3. a) Conception and farrowing rate, b) Number of pigs born, c) Retention rate to farrowing and weaning to parity 3 and d) total pigs born to parity 3 for gilts served and classified by estrus at service. From Pinilla & Patterson (2020). Different letters indicate significant difference between estrus at service classes (P < 0.05).
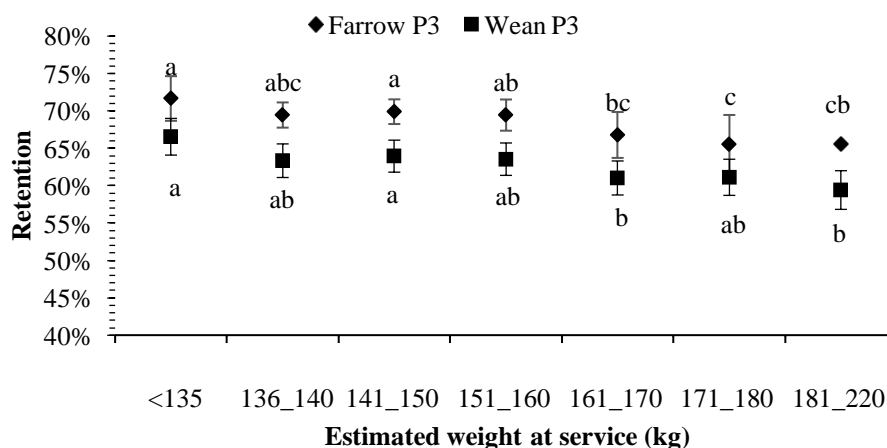
Figure 4. Retention rate to parity 3 for gilts served and classified according to weight at service. From Pinilla & Patterson (2020). Different letters indicate significant difference between weight at service classes (P < 0.05).

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An established Heat-No-Serve program permits effective pre-breeding management

Proper gilt acclimation and a positive metabolic state in the pre-breeding period optimizes first litter performance. As reported by Foxcroft & Patterson (2020), maintaining a high level of feed intake between first and second estrus in the gilt is critical for increasing ovulation rate at second estrus and thus increasing potential litter size born. This is based on earlier mechanistic studies that reported an immediate and negative impact of reducing feed intake in pre-pubertal gilts on tonic LH secretion (Booth et al., 1996). Any event that disrupts normal feed intake in the gilt will immediately impact LH secretion and remove the critical priming effect of LH secretion on follicular development and this lack of LH "priming" affects follicle and oocyte quality and gilt fertility. It is therefore important that after first estrus has been recorded, gilts should be acclimated to stalls or breeding and gestation housing at least 16 d prior to breeding (Foxcroft & Patterson, 2020b; Kraeling & Webel, 2015) and offered ad libtum feed and access to fresh water.

First litter size is predictive of later lifetime performance

Gilt management is a critical driver of SLP; gilts are the foundation of good production and drive farm success now and in the future. First litter size has been previously reported to be predictive of later lifetime performance (Gruhot et al., 2017; Iida & Koketsu, 2015; Koketsu et al., 2017) and our recent data confirms these results (Figure 5). Therefore, the implementation of effective gilt management programs that improve first parity litter size will have lasting effects on lifetime production and efficiency. The opposite is also true, poor management of the gilt prior to service limits the ability of sows to produce pigs in subsequent parities and to remain in the herd (Knox, 2019). Therefore, an integrated gilt replacement strategy is critical to the success of the breeding herd.
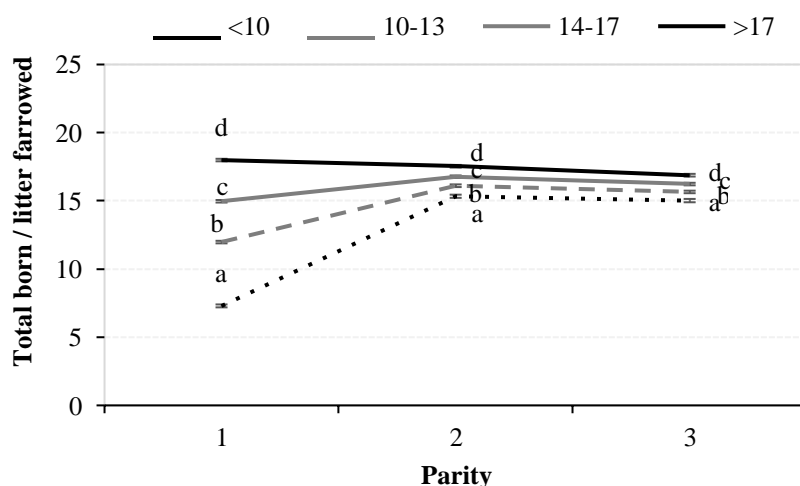
Figure 5. Data demonstrating that first litter size is predictive of total born in later parities in commercial sow populations. From Pinilla & Patterson (2020). Different letters indicate significant difference between litter size at first parity classes (P < 0.05).

Investing in the development and skills of stockperson is an integral part of the GDU

The success of a good gilt replacement program depends on allocating the necessary resources to the GDU. Two components of a successful gilt development unit are the development of the stockperson and ensuring the team has the tools needed to be successful. The importance of the position of a gilt development unit manager is often under recognized. As discussed throughout this paper, gilts are the foundation of the sow herd, and therefore, this role is critical to the success of the sow herd. It is essential to implement a program that includes a structured onboarding process, facilitated learning opportunities through training modules, goal setting and regular feedback and mentoring from management (from Pollman, 2021). It is also important to have dedicated GDU (BEAR) facilities and to provide your employees with the right tools to be successful. This will improve efficiency, job safety and employee morale.

Measuring and managing the key components of gilt development is critical

Large amounts of reproductive data are routinely collected and recorded in sow farm production databases. These records can be used to provide powerful insight to track and monitor reproductive success and to make data-driven

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decisions that will positively affect overall herd performance (Patterson & Foxcroft, 2019). Unfortunately, in the case of the replacement gilt, critical records such as age at puberty and heat-no-serve records are rarely collected and/or analyzed (Foxcroft & Patterson, 2020b; Koketsu et al., 2017; Patterson & Foxcroft, 2019). The four key records that we recommend to be recorded are age at puberty and estrus number, weight and age at service. As discussed previously, age at puberty is a benchmark for future fertility and gilts should have a recorded estrus by 200 days of age. Gilts should be bred on at least second detected estrus, between 135-160 kg and prior to 230 days of age. These key metrics of gilt development should be captured and reported on a weekly basis as illustrated in Figure 6. This report provides the stockperson a consistent measure to determine if the targets are being met on a weekly basis and provides an opportunity to troubleshoot suboptimal performance quickly. Furthermore, in the absence of the necessary gilt reporting in some sow management software, the use of data visualization and analytics provide powerful tools to track the gilt replacement program. Additional data that is also critical to track and trouble shoot reproductive issues include: Response dynamics of successive cohorts of gilts to puberty induction programs; Plots of the recorded inter-estrus interval; Reports on the outcomes of production tools such as PG600; Outcome response variables including conception and farrowing rates and litter size (total born, born alive, stillborn and mummies); Reports on SLP including lifetime productivity and retention. It is critical to use GDU-derived data to manage, analyze and monitor the performance of gilts.
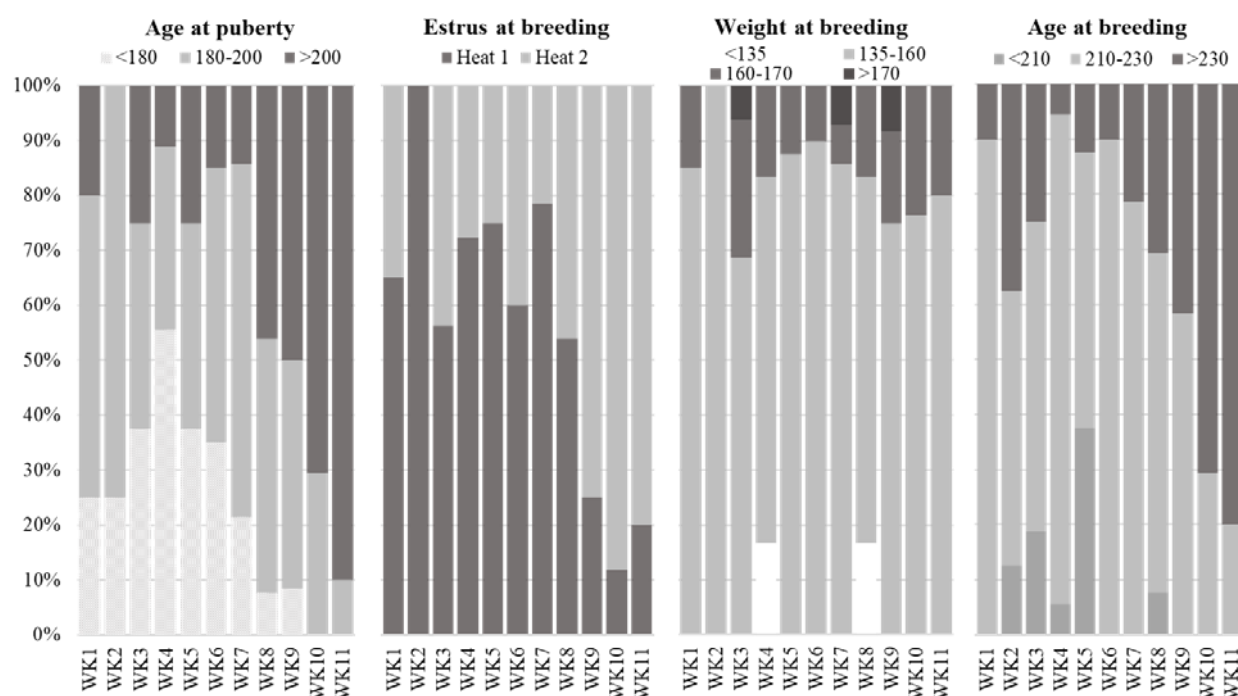
Figure 6. Key data that should be recorded as an integral part of gilt management programs.

Conclusion

In this review, we identified the key principles for improved sow lifetime performance. Each of the principles should be collectively viewed as necessary components of effective gilt development. The successful introduction and retention of gilts through the early parities drives lifetime performance of the breeding herd and represents an opportunity to improve and enhance overall production.

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Diagnostic Approach to Common Reproductive Problems on Farms

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Introduction

Problems associated with reproduction account for the majority of females being culled from herds worldwide. Reproduction is crucial to warrant profitability for wean-farrow sow farms and problems thus cause economical loses. Also, there is almost no farm that would not have "space" for improvement in reproduction (even though there aren't "problems"). Knowledge of available diagnostic tools and strategies are therefore crucial to reach/maintain reproduction targets or solve problems.

Common reproductive problems that the author has been encountered during his professional career include (in a non-prioritized order):
- Low conception rate
- Low farrowing rate
- "Early" fall-out
- Late fall-out
- Abortion
- High rate of returns
- Delayed puberty/High cull rate of gilts for "No Heat"
- Discharge/endometritis problem (puerperal = PDS & non-puerperal)
- Low litter size
- Long wean-estrus-interval

Any of these problems may have either a truly biological cause (i.e. sow and/or semen/boar failure; infectious or non-infectious), or is the result of management failure; the latter still being the number one reason for reproductive failures in females. Thus, any diagnostic work-up must include a careful management evaluation too.

When talking about "common reproductive problems" it means herd issues rather than individual problems. Each problem has its uniqueness, however, problems share similarities. "Diagnostic approach" always means to start a diagnostic work-up which depends on the case and comprises of joint but also specific diagnostic procedures. Identifying or verifying the problem based on a carful and comprehensive data record analysis has always to be the first step. This is not trivial as data entry differs between farms and is not always performed correctly. Once again, this record analysis will help to "narrow" the problem and "sharpen" the list of differentials. A farm-walk-through will follow and then a clinical examination of pigs that are specific to the problem (usually also involves pigs that are not affected as "controls"). The core element within the clinical examination may by ultrasonography by which the biological equivalent to the problem (but not necessarily the cause) can be identified. Other examinations may follow (e.g., postmortem with microbiology, mycotoxicology, virology; housing, feeding, semen analysis etc.). As always, the "cost-benefit equation" needs to be considered. A few examples of common reproductive problems will be elaborated below (please refer to e.g., Björkman et al., 2019, Kauffold et al., 2019, Althouse et al., 2019, Baumann et al., 2021).

Common reproductive problems

If gilts or weaned sows are not in heat, stimulation protocols, heat check procedures and personnel doing heat checking have to be evaluated. For instance, boar stimulation has been shown to be occasionally weak. Examples are in gilts where boars were either of reduced libido or "bored" at stimulation or were inadequately heavy scaring the gilts. In weaned sows, common mistakes are related to the time, frequency or intensity of boar stimulation (e.g., starting too late post weaning or performed only once a day). Since the phenomenon of "No Heat" is essentially driven by sexual maturity and/or functionality of the ovaries, this can be effectively assessed by ultrasonography. The problem (e.g., gilt vs. sows; delayed vs. silent) derives the time and frequency when ultrasound has to be done. In gilts it would make sense to scan animals at different age groups e.g., at 180, 200 and 220 days of age to confirm the problem and assess the amplitude of prepubertal vs. pubertal animals. If the problem is evident, it has then to be further diagnosed e.g., by body condition scoring (which may include also back fat (BFT) and muscle thickness (MT) measurement by

ultrasound). For instance, gilts at same ages but lower body weight as well as BFT and MT may display delayed or "No Heat" more frequently than better conditioned gilts. A case where gilts did not respond to an eCG treatment has been observed. In another case of gilts culled for "No Heat", an infectious cause has been suspected. In weaned sows with "No Heat", ultrasonography may be done at the time of projected breeding. Sows may have inactive ovaries (most often seen in primiparous sows with low body condition) or corpora lutea as a result of a lactational estrus (which turned out to be a common problem on German farms), but ovarian cysts are rather uncommon on a farm level. In the aforementioned situations, a postmortem examination of the genital tract is usually not required.

In cases of low conception or farrowing rate it is just logically to start the diagnostic work-up with controlling ovulation in order to determine when animals ovulate relative to breeding. A discrepancy of ovulation/breeding is one of the major reasons for low conception. However, if ovulation/breeding fits then checking for early embryonic losses by ultrasonography may follow. It is imperative to mention that, in the aforementioned cases, procedures used for pregnancy checking need to be carefully evaluated. While it is known that the return-to-service control (which is basically heat checking) is usually the least reliable procedure for pregnancy testing, ultrasonography can also fail. As an example, the author has been involved in cases of low conception/late term fall-outs where the person who did the scanning together with a poor ultrasound unit was identified as the source for the "failure" (i.e. open animals were misdiagnosed as pregnant and ended up as late fall-outs).

Low conception/farrowing rate with or without increased returns and occasionally also associated with vaginal discharge may, however, have other reasons. The time for sows while in lactation is crucial for the genital tract (especially the uterus) to involute. Involution is physiologically accomplished in the third week post farrowing. However, the process of involution can be negatively effected by e.g. high litter size, long parturition, hygiene failure, frequency of obstetrical interventions and many other factors. This may be associated with pathological puerperal vaginal discharge, which is, or is not accurately diagnosed by personnel, and thus may not be treated correctly (e.g. in terms of necessity and/or duration). As an effect, animals may not be cured when moved from lactation into breeding (i.e. may still have a "diseased" and maybe also bacteriologically positive uterus), and are thus highly susceptible to e.g. re- or superinfections (e.g. due to poor hygiene at breeding). Those animals are prone for conception failure, returns and eventually also the development of vaginal discharge. A careful audit of lactation is then always required, and ultrasound e.g. performed at breeding (see above) helpful to exclude ovulation/breeding failure. Also, animals that exhibited a lactation estrus and were bred in an estrus/ovulation synchronisation program (diagnosed as animals having corpora lutea at breeding; as observed in one case) may develop vaginal discharge also because of progesterone dominance at breeding knowing of its immunosuppressive activity. While ultrasound may be beneficial to identify problem animals it does always help always help to unravel the causes for the problem. A good example is the mycotoxin DON that is, like progesterone, immunosuppressive. Laboratory results based on submissions of genital organs of animals with reproductive failures such as low conception, returns and vaginal discharged demonstrated that DON may be a major player for such phenomena. Animals that displayed critical concentrations of DON in bile had often signs of a mild-severe chronic inflammation of usually multiple genital organs (e.g. cervix, vagina, oviduct, uterus), and were also multiply bacteriologically positive in uteri. Newer results indicate that the regional lymph nodes supplying the genital organs may also serve as a good specimen for the diagnosis of especially viral genital pathogens.

Conclusion

In conclusion, reproductive problems require a comprehensive diagnostic approach that depends on the specific problem. The same problem can have different causes. Understanding reproductive physiology is the bases of any diagnostic approach. Ultrasound is a very beneficial tool to pinpoint problems, but occasionally has to be complemented by additional examinations/analyses.

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Management of reproduction: piglet survival and fertility of the sow

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Introduction

During pig breed domestication, breeding has focused on lean tissue deposition, feed conversion efficiency, and above all, on prolificacy [1,2]. The larger the litter, the better the profitability for the farmer. Average litter sizes may have increased by 0.2–0.3 piglets/year [3]. However, increased litter size is associated with negative aspects such as high energy demand for milk production [4], prolonged farrowing duration [3], and pre-weaning mortality [5]. Thus, increased litter size negatively affects metabolic and reproductive health of the sow, and survival and health of the neonates.

Based on 20 different studies carried out between 1990 and 2019, litter size has increased from ca. 10 to 20 piglets and farrowing duration has increased from 1.5-2 to 7-8 h (Figure 1; [3, 6]). While the described tendency is subject to differences in breeds and farrowing housing environments, the overall tendency is therefore rather worrying. The extended duration of farrowing appears as an outcome of intensive breeding for prolificacy in the pig [3].
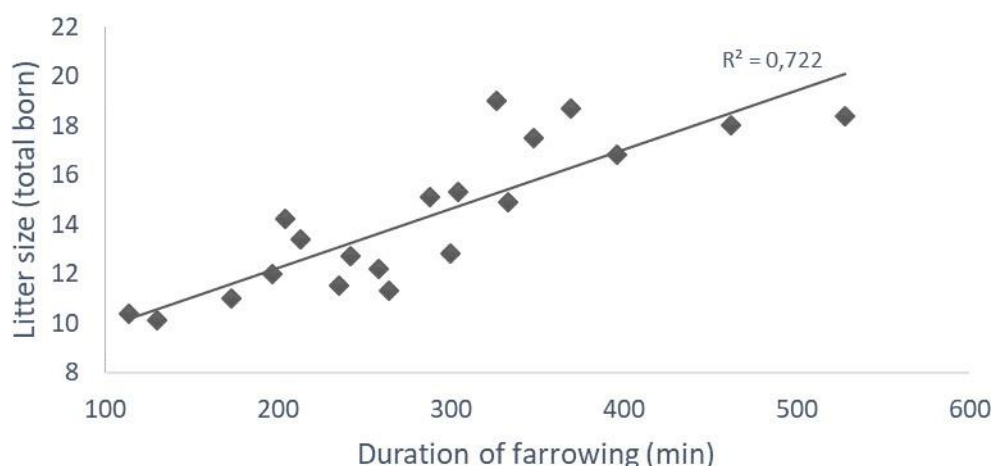
Figure 1. Increased farrowing duration with increased litter size (a conclusion based on 20 studies on farrowing duration [3])

The increasing litter size and prolonged farrowing present an immunological challenge for the sow and the newborn piglets [3,7]. A healthy female experiences an inflammatory state during parturition with pro- and anti-inflammatory pathways being in balance [8]. It seems that in modern hyperprolific sows, this balance is, already before parturition, disturbed and the pro-inflammatory state prolonged [9,10]. A prolonged pro-inflammatory state and stress, put sow into an immune–endocrine disequilibrium which causes negative effects on metabolism, the endocrine systems and the immune system, making the sow susceptible for birth complication, reduced colostrum and milk production, and post-partum disease [8-11]. This is also problematic for the piglets of these sows, because birth complications and post-partum diseases with decreased colostrum and milk production have been linked with increased piglet mortality and decreased piglet development [12].

Further, with prolonged farrowing, the last 20–30% of the foetuses to be born seems not to have access to high-quality colostrum, as its quality (i.e. immunoglobulin G [IgG]) rapidly declines after the onset of parturition [13]. They also have less time to suckle on colostrum due to a decreased opportunity for colostrum intake, increased competition for teats, and reduced birth weight [3]. These factors may result in reduced immunity and the emergence of diseases during the growing phase of piglets/fattening pigs.

The metabolic challenge related to the hyper-prolific sow production model begins during gestation and proceeds beyond farrowing and lactation. The sow is supposed to eat enough to meet the nutrient requirements of growing litters prior to farrowing, which may cause some of the problems seen around the time of farrowing [14,15]. During the early stage of lactation, sows with large litters loose more energy while producing more milk that cannot match

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up with the energy from their feed, ending up in a negative energy balance (NEB; [16,17]. Negative energy balance impacts follicle development after weaning [18-20], oocyte quality [18,19], embryo development [16], and piglet birth weight [21]. Thus, pre-mating diets or optimizing the sow metabolic state during lactation may be options for improving subsequent sow fertility. It is therefore important to review management strategies around reproduction and piglet survival in large litters.

Piglet colostrum intake and mortality

Piglets' first suckling and colostrum intake

Piglets' first suckling behaviour is the most important factor for colostrum intake, which is crucial for their survival and growth. Studies have shown that the average time of first suckling ranged from 27 to 62 min [22-27] and the interval from udder touch to first suckling averaged 9 min [26]. Other studies showed that the times of the first udder contact (range from 4 to 215 min) and colostrum intake (range from 0 to 116 min) also varied among individual piglets [25,26]. The piglets' first suckling behaviour depends on piglet characteristics such as body weight, size and vitality [23,28]. If piglets take a longer time until first suckling, they experience more heat and energy loss, lower colostrum intake and a higher mortality rate during lactation [23]. Thus, the physical characteristics and vitality of piglets can play a crucial role in their survival and growth.

The energy requirement of newborn piglets is very high because of high physical activity and thermoregulation directly after birth [29,30]. Piglets acquire energy mainly from colostrum [13,31], which is mainly composed of moisture, protein, fat and lactose [31,32]. The energy content (e.g. fat and lactose) of colostrum has a major impact on short-term piglet survival during lactation [13]. Colostrum also contains a high concentration of IgG [32,33], which is essential for piglet immune systems and thereby for their long-term survival during lactation [34]. The composition of colostrum changes nearly hourly. Theil et al. [13] showed that during the first 24 h after birth, lactose content increased from 3.5 to 4.4%, fat content increased from 5.1 to 6.9%, and energy content increased from 260 to 346 kJ/100g. The concentration of IgG, on the other hand, decreased rapidly by 50% during the first 6 h after birth of the first piglet [35] and continued to decrease further during farrowing and until 24 h after farrowing (e.g. 62.3 vs. 16.8 mg/ml, respectively for at birth and 24 h after birth [36]). In modern sows with large litters, changes in energy and IgG content in colostrum are also similar to those of sows with relatively small litter size despite the increases in litter size and duration of parturition [3,13,37]. In terms of optimizing energy intake, late colostrum (around 12 h after farrowing) therefore seems more advantageous compared to early colostrum [13]. On the other hand, early colostrum may play a more crucial role in the passive immunity of piglets than late colostrum [32]. Piglet colostrum intake has been shown to positively relate with weaning and inversely related with pre- and post-weaning mortality of the piglets [38]. Declerck et al. [39] and Hasan et al. [40] reported that the colostrum intake of each additional piglet in a large litter decreased by approximately 9 g. This could be due to a limited colostrum yield from the sows [40] and increased competition within litters [41]. Colostrum also contain bioactive factors such as insulin, epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) [42], which are beneficial for piglet growth and survival. Considering that the energy mobilisations during late gestation are prioritised for mammary growth and colostrum production [13], feeding strategies focusing on late gestation can be one option for improving sow colostrum yield. Also, providing energy source to piglets right after birth has been recommended from many studies and will be discussed below. Therefore, to optimize sow colostrum yield and piglet colostrum intake, nutritional management during late gestation and lactation should be considered more carefully in large litters.

Factors increasing piglet mortality in large litters

Increased mortality in large litters is of considerable economic and welfare concern in modern pig farming. High pre-weaning mortality in large litters may result from decreased piglet birth weight and increased within-litter birth weight variation (i.e. litter uniformity; Table 1) [5,43,44]. Correspondingly, the number of piglets weaned has not perfectly matched with increased litter size. Recent studies showed that total pre-weaning mortality, including stillbirths, ranged from 13 to 15% in large litters [45–47]. In severe case, sows kept under risky conditions with a large litter of an average 19 piglets have 17.9% of piglet mortality during the first day of lactation in open farrowing crate [25]. Among pre-weaning mortality, 72 h of postnatal life is the most critical period (for review, see [49]). The great majority of piglet mortality is caused by crushing, starvation and hypothermia [49]. In particular, starvation and hypothermia, which can be derived mainly from piglet characteristics, may cause piglet crushing and death during lactation [50]. Low birth weight in piglets may be linked to lower vitality/viability [51], a longer time to the first suckle [25], and less ability to compete for colostrum intake with littermates (for a review, see [39]). Moreover, limited capacity to ingest colostrum of low-birth-weight piglets [52] could be one of the reasons for impaired colostrum intake [53]. Furthermore, Baxter et al. [23] have demonstrated that piglets that die before weaning had lower birth weights and lower rectal temperatures at birth and 1 h after birth compared to piglets that survived. This may imply that

hypothermia can also be an important mortality factor in low-birth-weight piglets. Indeed, Herpin et al. [30] showed that smaller piglets may experience greater heat loss and thus a decreased ability to thermoregulate when compared to larger piglets. Considering that low-birth-weight piglets showed higher mortality, especially during the first 24 h after birth [54,55], certain supportive management routines around parturition will be needed in the management of large litters will be discussed.

Table 1. Regression coefficients (β) between the number of total piglets born and litter characteristics at birth in sows.

| | Total number of piglets born, n | | | |
	Milligan et al. [40][1]	Wienjtes et al. [5][2]	Wientjes et al. [17][3]	Han et al. [41][4]
Litter characteristics				
Mean birth weight, g	-46***	-40***	-41***	-37***
CV of birth weight	0.39***	0.76***	0.83***	0.60**
Piglets < 1,000 g, %	-	2.4***	1.9***	2.0***

[1] Conventional YL sows, 10.7 total born piglets (n = 4,222).
[2] Organic Topigs20, 17.4 total born piglets (n = 1,864).
[3] Conventional Topigs20 and Topigs40 sows, 13.5 total born piglets (n = 2,128).
[4] Conventional DanAvl sows, 19.1 total born piglets (n = 1,065).
** $p < 0.01$, *** $p < 0.001$

Litter uniformity, in addition to individual birth weight, can be a major factor affecting piglet mortality. Increased litter size resulted in poor litter uniformity, which elicited a higher proportion of small piglets (< 1 000 g; Table 1) [5,21,44]. Results by Wientjes et al. [5] support this finding, as they showed the coefficient of variation (CV) of birth weight to positively relate to mortality during the first three days after birth in large litters. Furthermore, poor litter uniformity (i.e. large variation of within-litter birth weights) resulted in less colostrum yield by sows [53] and unevenly distributed colostrum intake by piglets (reviewed by [43]). Poor uniformity at birth causes not only high mortality but also poor uniformity at weaning [44,55]. Thus, improving litter uniformity, either by pre-mating nutritional strategies or breeding, is of great interest with regard to large litters.

Stillborn piglets are also of great concern in large litters. Generally, stillborn rates in piglets have been in the range of 5–10% in recent studies (reviewed by [56]). Stillborns can be classified into two types, depending on their time of death [57]. Piglets in one group die before parturition (antepartum or prepartum death; type 1), while piglets in the second group die during parturition, which represents a great majority of all cases (intra-partum death; type 2; [58]). Increased farrowing duration with higher litter size (Figure 1) may increase type 2 stillborn rates. Canario et al. [59] reported a potentially higher risk of stillborn piglets with a litter size of more than 14 piglets. A recent study also found that a higher stillborn rate was related to larger litter size [60], which is in accordance with earlier studies [61,62]. This may be explained by the greater risk of asphyxiation after detachment of the placenta [63], possibly due to prolonged exposure to uterine contraction due to increased farrowing duration.

Feeding strategies for improving piglet survival

Based on the findings of high mortality in large litters, management strategies for increasing piglet survival rate should focus on strategies applicable during late gestation and before parturition and strategies applicable after birth. In the review of Theil et al. [13], they addressed the importance of sow nutrition in late gestation on colostrum yield and composition. Briefly, different dietary composition during late gestation may alter both colostrum yield and quality. Before parturition, high-fibre diets seems to result in an improved farrowing process [15,64] and colostrum production [13], and thereafter in reduced pre-weaning mortality [64]. Frequent daily meals (more than thrice daily) before farrowing are recommended for improving both the energy status and farrowing process of sows with large litters [65]. For example, Feyera et al. [65] observed that sows with a shorter time from the last meal until the onset of farrowing had a shorter farrowing duration, less probability of requiring farrowing assistance, and a low number of stillbirths. This finding may suggest that decreasing serum glucose levels may be one of the mechanisms through which farrowing duration is prolonged.

Dewey et al. [66] found that farms that provided oral administration of colostrum or glucose to piglets and performed split-nursing showed higher survival rates compared to farms with less intensive management. Especially for weak piglets, helping to establish breathing, assisting them in reaching the udder, and keeping them warm may also be recommended, as suggested by Herpin et al. [63]. These management routines can reduce the time of first suckling [24,63,67], thereby leading to an increase in colostrum intake and survival rate. Vasdal et al. [24] stressed that drying piglets and placing them onto the udder of the sow directly after birth is a key point for optimizing neonatal survival in large litters. They found less than 10% mortality (of total born) in a litter with over 15 total piglets in the open-farrowing system with intensive piglet management routines. This mortality rate is indeed low when compared with

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a mortality rate of 17.9% observed during the first 24 h after birth in litters of hyper-prolific sows that had not been given management routines at birth [25].

Providing energy supplementation to small piglets by hand has also been recently studied as a means to cope with the insufficient energy intake of piglets in large litters [45,68–71]. Declerck et al. [68] showed that pre-weaning mortality was reduced when small piglets were fed with energy supplementation (e.g. soy oil and coconut oil) directly after birth. Glycerol-rich supplementation and colostrum replacers also seemed to be beneficial for small piglet survival [71]. On the other hand, some studies did not find an increased survival rate with energy supplementation (sow colostrum and coconut oil) [46,70]. Thus, both drying piglets and providing them with energy supplementation, and thereafter moving them to the sow's udder may be the most effective management routines for optimizing piglet survival in large litters.

Sow lactational body condition loss and subsequent fertility

Lactational body condition loss and follicle development

Sows lose their body condition mostly during lactation. The losses consist of both protein and lipid. In practical situations, backfat thickness (BF) is widely measured to predict sow lipid status. Loin muscle depth (LM), which represents protein status, contains relevant information on sow metabolic state and reproductive performance, especially if lean sow lines are used for breeding [16,19,20]. The increased number of suckling piglets in large litters resulted in sows being in severe NEB (attributed to the loss of proteins, lipids, or both) during lactation [4]. This is caused by the high metabolic demands for milk production [72]. Severe NEB (e.g. approximately 10–12% body weight loss) may compromise subsequent fertility, causing e.g. extended weaning-to-oestrus intervals (WEI), lower pregnancy rates, and lower subsequent litter size [73]. In modern hyper-prolific sows, however, severe NEB appears to associate with a lower ovulation rate or embryo survival rather than extended WEI (reviewed by [74]).

Impaired ovulation rate or embryo survival can be explained by compromised follicle development at weaning. Severe NEB resulted in smaller follicle diameter at weaning [18–20,75]. This may originate from the detrimental effect of NEB on luteinizing hormone (LH) and follicle development. In early lactation, LH is suppressed by sucking-induced inhibition of the GnRH (reviewed by [76]). As lactation progresses, LH pulsatility is normally restored [77], which stimulates follicle development. However, sows with low feed intake had lower LH pulsatility and smaller follicles at weaning compared to sows with high feed intake during lactation.

In large litters, follicle diameter at weaning is approximately 4-5 mm [19,20]. After weaning, pulsatile GnRH release may induce the release of both LH and follicle-stimulating hormone (FSH), which are important for follicle recruitment, growth and ovulation [78]. As a result, follicles grow to reach the pre-ovulatory size (7–8 mm) [20,79,80] usually within seven days after weaning (reviewed by [74]). Smaller follicle diameter at weaning is related to longer WEI and weaning-to-ovulation interval (WOI) [20,81–83]. This is because smaller follicles take more time to reach the pre-ovulatory phase [82], after which oestrogens produced by pre-ovulatory follicles result in oestrus and ovulation (reviewed by [81]).

Further, sow metabolic state may represent the follicular fluid metabolic state, as follicular fluid can be considered an exudate of sow blood. In the study by Costermans et al. [19], plasma IGF-1 level, which is negatively related to sow body condition loss during lactation [19,20], was strongly related to follicular fluid IGF-1 level after weaning. As IGF-1 is important for follicle and oocyte development, ovulation, and embryonic development and implantation [19,20], the importance of sow metabolic state on reproductive performance and subsequent litter characteristics seems to be clear.

Follicle development and subsequent fertility

A schematic drawing of the relationship between sow NEB during lactation and litter uniformity at subsequent parturition is described in Figure 2 [5]. This may be explained by the detrimental effect of sow body condition loss on follicle development and subsequent fertility. Follicle development before ovulation plays a major role in oocyte quality, embryo development and, eventually, piglet characteristics at birth in sows (reviewed by [84]).

Studies have shown that impaired follicle development at weaning can result in a compromised follicle pool before ovulation [75] and a lower oocyte maturation rate [18,19]. Further, there is a positive relationship between follicle diameter at ovulation and corpus luteum (CL) diameter after ovulation [79,85]. Good CL development is necessary for embryo development during early pregnancy [2,86,87], as CL has been shown to positively relate with progesterone level and pulse [88–91]. Smaller follicles at ovulation may therefore be detrimental for early embryo

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development. Considering that piglet characteristics are largely determined at the early embryo developmental stage [92], we suggest that follicle diameters at weaning may also be related to piglet characteristics. Likewise, the heterogeneity of the follicle pool before ovulation may have an impact on litter uniformity at birth with a similar mechanism (reviewed by [74]).
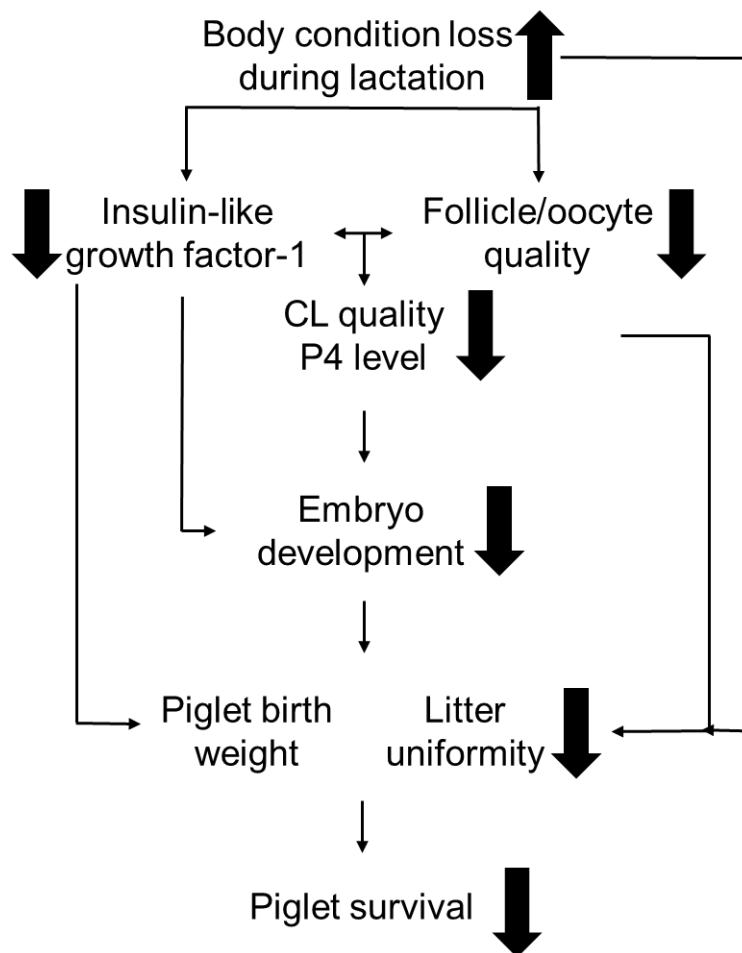
Figure 2. Schematic illustration of body condition loss during lactation and the IGF-1 level, follicle/oocyte quality, embryo survival, and litter characteristics and its consequences for piglet survival.

Insulin-like growth factor-1 (IGF-1) is a possible mediator affecting follicle and oocyte development. It is very important in follicular fluid, as it can bind to IGF-1 receptors on the oocytes and granulosa cells. Once bound, it may synergize with FSH so as to activate follicular growth, steroidogenesis, and the oocyte cleavage rate [93–95]. A recent study also found that IGF-1 in the follicular fluid is positively related to follicle diameter before ovulation [19]. During WEI, sow plasma IGF-1 level is strongly related to the follicular IGF-1 level [19] and its levels at weaning are positively related to those during WEI [20,96,97]. Thus, higher IGF-1 and larger follicles at weaning appear to favour higher oocyte quality. The IGF-1 level around ovulation is also positively related to CL diameter and the increment of progesterone level after ovulation [98], and to embryo survival during early pregnancy [16]. Our recent study observed that higher plasma IGF-1 before ovulation (at oestrus) was positively related to piglet mean birth weight [44]. Thus, IGF-1 -mediating follicle development, which was affected by NEB [19,20], has a major impact on subsequent sow fertility. In addition, extracellular vesicles may be among further mechanisms through which NEB-driven reduction in follicle development can affect the developing ova within the follicle, as shown for canines in vitro [99].

Embryonic mortality in large litters

As a consequence of breeding for a large litter, the ovulation rate (OR) has increased and is currently approximate to 25–30 (reviewed by [74]). Embryonic and piglet mortality have increased with increased OR [100,101]. However, the number of piglets could only increase to a certain limit because of the higher embryonic mortality associated with increased OR (reviewed by [74]). Early embryonic mortality occurs before implantation (around 12 or 13 days of gestation), while late embryonic mortality occurs after implantation between 13 and 35 days of gestation. In sows,

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early embryonic mortality increased with increasing OR and was approximately 59% of the total embryonic mortality [100]. Embryonic heterogeneity within litters may be a major reason for early embryonic mortality. Less-developed embryos cannot develop further in a uterine environment, which is advanced by the more-developed embryos (reviewed by [74]). In detail, oestradiol produced from more-developed embryos stimulates uterine secretions for their own implantation but this results in an unfavourable environment for less-developed embryos [102,103]. Synchrony between developing embryos and the uterine environment is important for successful implantation. Embryos lagging behind in development may experience an uterine environment that is asynchronous with their own development and implantation may therefore fail [104]. Considering that embryonic heterogeneity is largely affected by follicle heterogeneity [101,105], the importance of follicle development before ovulation is once again highlighted. However, increased OR also seems to associate with compromised follicle development. Sows with increased OR showed decreased CL diameters, which were derived from a decreased follicle diameter [79,85]. This implies that breeding for a large litter likely contributed to compromised follicle development. Although less-developed embryos may survive through the implantation process, they may be more vulnerable to dying later during gestation. Late embryonic mortality was ca. 42% of the total mortality and it also increased as OR increased [100]. Limited uterine capacity and competition for space and/or nutrients are major reasons for late embryonic mortality (reviewed by [103]). Da Silva et al. [100] showed that embryos with small size and small implantation sites had higher mortality at a late stage of pregnancy. The small size of the implantation site can be linked to a small placental site [106], which may be harmful to foetal development.

Management routines during/after lactation for subsequent fertility

Only five or six days of WEI appears too short to recover from severe NEB in hyper-prolific sows and to support their follicles in reaching the pre-ovulatory size and high-quality oocytes. Thus, skipping the first heat and inseminating at the second oestrus may be recommended for sows with severe body condition loss during lactation. This recommendation stems from the study showing that a longer weaning-to-pregnancy interval (WPI; > 21 day) resulted in better litter uniformity (i.e. lower SD and CV at birth weight; [21]. Wientjes et al. [21] explained that this may be due to the longer recovery of metabolic states and the restoration of follicle development, which is beneficial for subsequent fertility.

Pre-mating diets are one option for stimulating follicle and oocyte development. A fibre-rich pre-mating diet (e.g. sugar beet pulp) before ovulation can have a positive impact on oocyte quality and maturation in the gilts [107]. Furthermore, supplementing insulin- or IGF-1-stimulating diets (dextrose and lactose) during lactation and WEI can improve litter uniformity [108,109]. Nevertheless, only a few nutritional factors have been evaluated as components of pre-mating diets. Considering that sow IGF-1 levels after weaning are positively related to pre-weaning levels [20,97], pre-mating diets during the late or whole lactation period may prove effective. Optimizing sow metabolic state during lactation is also recommended. This may be done by identifying the ideal feed composition of lactation diets, such as protein and amino acids levels, especially in a hyper-prolific situation.

Conclusions

Large litters do not come without a catch. Increased litter size creates problems with piglet survival during lactation and sow reproduction that need addressing. Large litters only occur through increased ovulation rates. These rates are associated with compromised follicles that appear to negatively affect early embryonic development and pregnancy-supporting mechanisms such as CL development. These impaired developments result in increased embryonic and foetal mortality. At the end of pregnancy, the process of parturition also seems tightly linked with litter size. Increased litter size prolongs the process of parturition, leaving a proportion of the litter with reduced chances for suckling high-quality colostrum for a reduced period of time under increased competition. Farrowing, early lactation management procedures and late lactation nutritional management are keys to tackling the increasing problems associated with large litters. In particular, nutritional management of the sow around the end of lactation, involving IGF-1-driven follicle development seems important.

Abstract

As a result of intensive breeding, litter size has considerably increased in pig production over the last three decades. This has resulted in an increase in farrowing complications. Prolonged farrowing will shorten the window for suckling colostrum and reduce the chances for high-quality colostrum intake. Studies also agree that increasing litter sizes concomitantly resulted in decreased piglet birth weight and increased within-litter birth weight variations. Birth weight, however, is one of the critical factors affecting the prognosis of colostrum intake, and piglet growth, welfare, and survival. Litters of uneven birth weight distribution will suffer and lead to increased piglet mortality before

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weaning. The proper management is key to handle the situation. Feeding strategies before farrowing, management routines during parturition (e.g. drying and moving piglets to the udder and cross-fostering) and feeding an energy source to piglets after birth may be beneficial management tools with large litters. Insulin-like growth factor 1 (IGF-1) -driven recovery from energy losses during lactation appears critical for supporting follicle development, the viability of oocytes and embryos, and, eventually, litter uniformity. This paper explores certain management routines for neonatal piglets that can lead to the optimization of their colostrum intake and thereby their survival in large litters. In addition, this paper reviews the evidence concerning nutritional factors, particularly lactation feeding that may reduce the loss of sow body reserves, affecting the growth of the next oocyte generation. In conclusion, decreasing birth weight and compromised immunity are subjects warranting investigation in the search for novel management tools. Furthermore, to increase litter uniformity, more focus should be placed on nutritional factors that affect IGF-1-driven follicle development before ovulation.

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Antimicrobial use in pig production in Europe: Less is more

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Introduction

Antibiotics knew their first applications in humans, revolutionizing the practice of medicine and gaining early on the nick name "miracle drugs". At the beginning of the 20th century, before their discovery, half of all deaths in the United States were related to infectious diseases. At the dawn of the 21st century, the burden of infectious diseases had become relatively limited compared to non-infectious causes. Besides, antimicrobials are important catalysts for medical improvements. Without them, many achievements in modern medicine, such as major surgeries, organ transplantation or cancer chemotherapy, would become impossible, as they would imply the same high risks due to infections as in pre-antibiotic times (Cars et al., 2008; Laxminarayan et al., 2013).

The discovery of antimicrobials also had their impact on Veterinary medicine, adding to a better health and welfare in animals (Economou & Gousia, 2015). Penicillin had its first application in dairy cows, where it was used to treat mastitis (Johnston, 2001). Treatments like the latter can be administered individually, which is mostly the case in companion animals, dairy cattle, horses and breeding pigs. However, when larger groups of animals need to be treated such as in poultry or swine, group treatments are often provided orally via feed or water (Schwarz & Chaslus-Dancla, 2001). When such a group medication is initiated because of clinical signs in only a subgroup of the animals, this is called a metaphylactic treatment. When it is administered to prevent problems at critical stages in the production phase (e.g. at weaning), in groups of animals not showing clinical signs, these administrations are prophylactic. The discovery and early use of antibiotics in animal production coincided with the post-war revolution from small scale extensive livestock production systems to a more intensive industrialized livestock production, where animals were housed indoor in large flocks and herds (Gustafson & Bowen, 1997; Steinfeld, 2004). This evolution not only resulted in farmers using more metaphylactic and prophylactic medication, it also paved the way for the commercial application of antibiotics as growth promotor.

The ban on growth promotors in Europe

Earlier studies on the impact on production of antibiotics given in the feed as growth promoters indicate productivity gains ranging from 1% to double digits, depending on factors such as nutrition, breeding, housing, sanitation, as well as husbandry and management practices (Whalstrom et al., 1956; Hanson et al;, 1955; Barber et al., 1958). However, recent studies have concluded that the current productivity benefits from the use of antimicrobial growth promotors (AGPs) in the feed have declined as the result of the adoption of modern production and management practices (Laxminarayan et al., 2015). Hence, AGPs have benefited poor management systems but they should have no place in modern animal production as they promote antimicrobial resistance (AMR). Indeed, the use of AGPs gradually phased out in Europa and, in 2003, the EU decided to ban all AGPs by 2006 (EC, 2006; Wielinga et al., 2014). In contrast to some expectations, this did not result in a substantial decline in food animal production in Europe. Although the first ban in Sweden did lead to some initial animal health problems, this was successfully addressed by improved management and disease prevention (Swedish Ministry of Agriculture, 1997). The lessons learnt from the Swedish experience also helped other European countries to cope with the subsequent EU-wide ban that was applied more gradually. In Denmark and the Netherlands, a shift towards increased therapeutic antimicrobial use (AMU) was also observed after the ban of AGPs. However, this increase turned out to be only temporary and was even non-existent in other countries such as Norway (Bos et al., 2013; Grave et al., 2004; 2006).

Quantitative insights in antimicrobial use in pigs in Europe

Surveys on antimicrobial use in pigs in Europe

Since the ban on AGPs in Europe focus has shifted to the therapeutic, metaphylactic and prophylactic use of antimicrobials in animals. This has resulted in a number of surveys in European countries describing AMU in pigs, both quantitatively and qualitatively. These surveys were conducted in Belgium (Timmerman et al 2006, Callens et al

2012); Denmark (Jensen et al., 2011; Nielsen et al., 2021), Spain (Moreno et al., 2014), Germany (van Rennings et al., 2015; Hemme et al., 2018), Sweden (Sjölund et al., 2015), France (Hemonic et al., 2018), Italy (Scopetta et al., 2017; Scali et al., 2020), Ireland (O'Neill et al., 2020) Finland (Yun et al., 2021; Sali et al., 2021) and Switserland (Echterman et al., 2020)

These surveys have been a crucial step towards a more detailed understanding of AMU and its risk factors in pig production. Many of these studies described huge differences in AMU over the course of the production cycle, with the majority of the use in young pigs. Other typical findings include a large variation in AMU between farms within the same country and the frequent application of prophylactic medication, often with important contributions of critically important antimicrobials such as 3° generation cephalosporin's and fluoroquinolones. In addition, farm size, veterinarian, poor biosecurity and farm health management, have all been described as drivers for AMU.

Some of the studies also were longitudinal in nature and provided evidence for the possibility to reduce AMU over time, without necessarily jeopardizing animal health and productivity (Postma et al., 2017; Collineau et al., 2017). This was in contrast to what was often claimed before, namely that high AMU would be necessary to support intensive production and that reducing AMU would result in lower production outputs.

A limitation of the described surveys is the huge variation in metrics used to quantify antimicrobial use, hampering comparisons between countries (Postma et al., 2015; Collineau et al., 2017). Therefore, the execution of multi-country studies, using the same quantification methodologies was a big step forward. A first multi-country study (France, Germany, Sweden and Belgium) was described by Sjölund et al. (2016). In this study, it was found that weaned piglets received the most antimicrobials, followed by suckling piglets. Furthermore, it was observed that AMU was significantly associated across age categories, indicating that farms with a high use in piglets also used more antimicrobials in their finishers. This may, among other things, be explained by farmers' habits and behaviour (Visschers et al., 2016). But above all, the study showed surprisingly large differences in AMU between the countries included. These differences between countries, but also between herds, might be related to the differences in disease prevalence or differences in the level of biosecurity. However, they may also reflect variations in rules and regulations in the countries and/or a certain attitude towards AMU of farmers and veterinarians not necessarily linked to the true animal health situation.

In a second multi-country study, AMU data on group medication to a single batch of finisher pigs from birth to slaughter were collected at 180 pig farms in nine European countries (Sarrazin et al., 2019). AMU was quantified using the treatment incidence (TI) indicator and was based on the defined daily doses (DDDvet). DDDvet values are technical units of AMU measurement provided by the European Medicines Agency (EMA). TI_{DDDvet} represents the percentage of time a pig was treated with antimicrobials in a certain production phase or its entire life (Timmerman et al., 2006). The majority of antimicrobials were administered to weaners (69.5% of total TI_{DDDvet}) followed by suckling piglets (22.5% of total TI_{DDDvet}) (Figure 1).
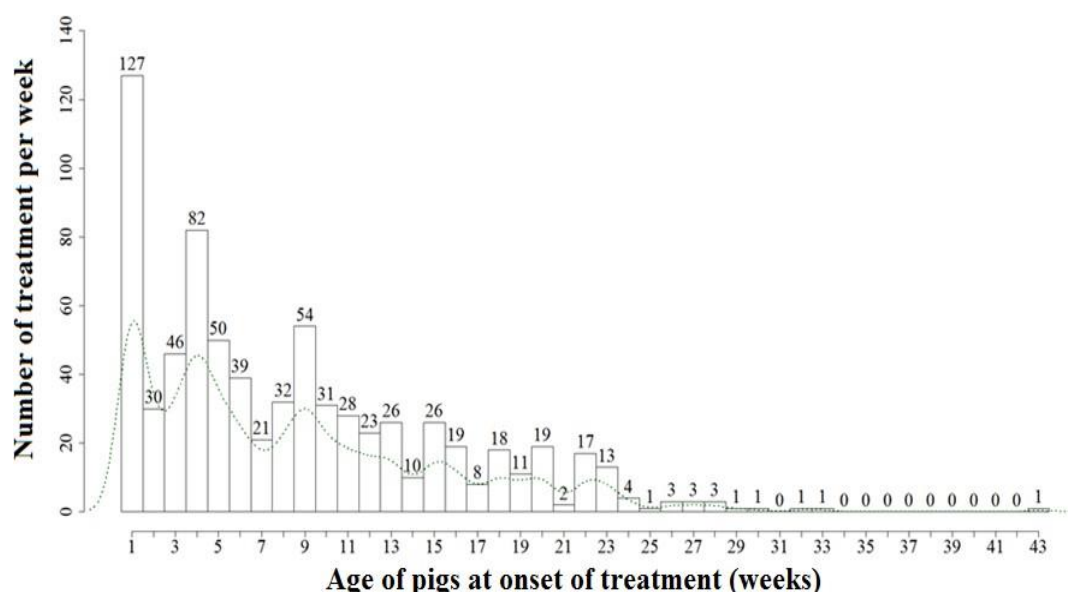
Figure 1: Histogram showing the number of antimicrobial group treatments per week applied to a batch of pigs from birth to slaughter based on 750 treatments (30 instances of treatments missing). The green dotted line represents the weekly average treatment incidence (adapted from Sarrazin et al., 2019).

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In this multi-country study AMU varied again considerably between countries and farms with a median TIDDDvet of 9.2 for a standardized rearing period of 200 days (Figure 2). This means that the median pig in this study received antimicrobials during 9.2% of its lifetime from birth to slaughter, or approximately 18 days in total. The fact that no group treatments were reported in either suckling piglets, weaners or finishers during the observed rearing period in 11.7% of the farms, showed that it is possible to rear pigs without systematic use of antimicrobials. The large variation in quantitative and qualitative AMU between farms shows that there is still room for improvement towards responsible AMU. For instance, one could reduce AMU at strategic time points in combination with increased biosecurity and a restricted use of the highest priority critical important antimicrobials.
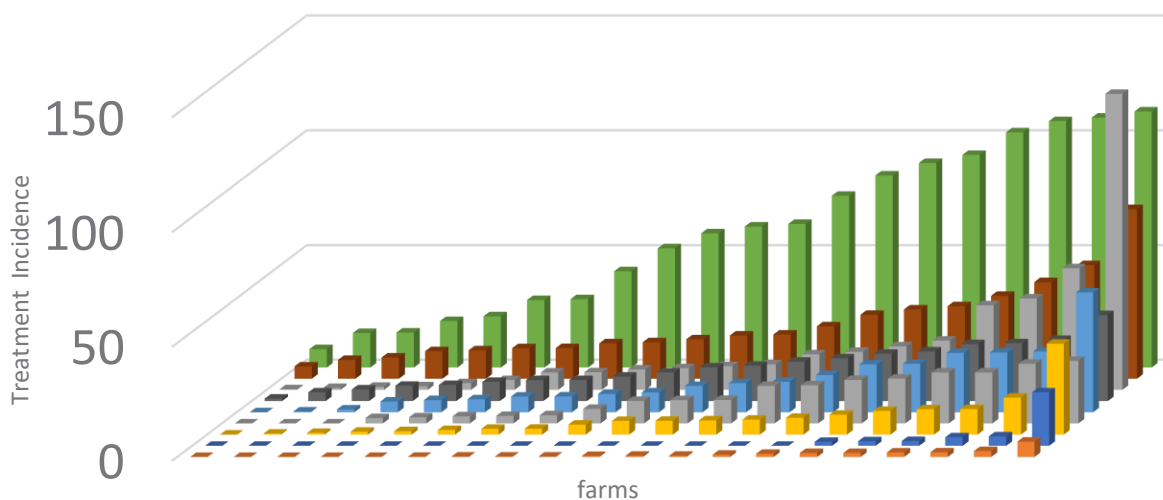
Figure 2: Country-level comparison of TI per 100 pig-days at risk, based upon group treatment data for a standardized lifespan of 200 days (TI200) based on defined daily doses (DDDvet). Every colour represents a different European country included in the study (adapted from Sarrazin et al., 2019).

Extended-spectrum penicillins (31.2%) and polymyxins (24.7%) were the active substances most often used in group treatments, with the majority administered through feed or water (82%) although considerable difference was observed regarding the administration route between countries. In one country 59% of all treatments were parenterally whereas in another country this was only 7%. The average treatment duration for parenterally administered antimicrobials was 2.6 days (minimum 1; median 3; maximum 14) for non-long acting (LA) formulations and 5.2 days for LA formulations, while for oral treatments this was 10.6 days on average. Moreover, 10% of the oral treatments were applied for a period of at least 21 consecutive days. Also in this study higher AMU at a young age was associated with higher use in older pigs. The most frequent indications for treatment were general (37.5%), intestinal (24.4%) and respiratory disorders (20.1%).

Systematic monitoring of sales data

Next to the many surveys conducted in different European countries, EMA launched the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project in 2005. In this project, AMU is being quantified in Europe at country level based on national sales data of veterinary antimicrobials. The last report (data up to 2020) provides information on the sales of antimicrobials in 31 European countries (Eleventh ESVAC report, 2021) (Figure 3). The use is expressed in milligrams of active component sold in relation to the Population Correction Unit (PCU), which is a proxy for the biomass of the food-producing animal population (including horses) in a country.

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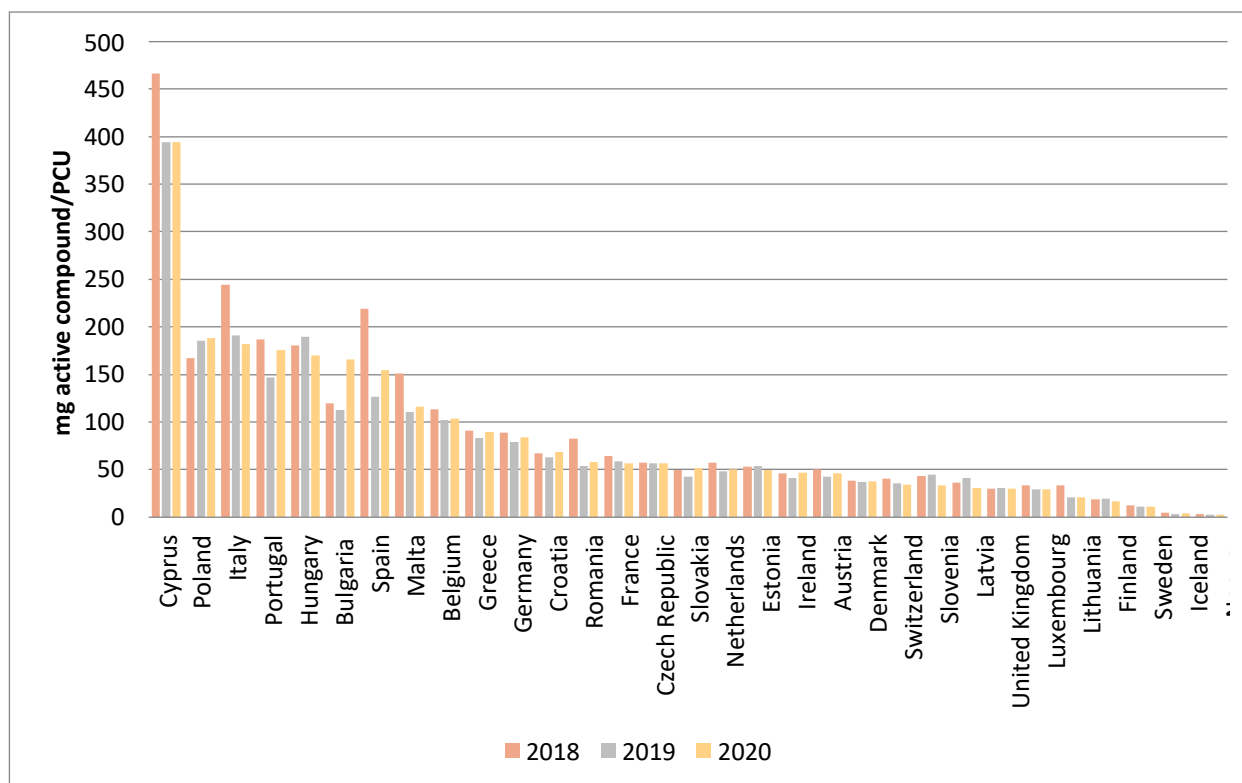
Figure 3: Antimicrobial use in animals in 31 European countries expressed in mg active component/ PCU between 2018-2020 (based on eleventh ESVAC report, 2021).

The ESVAC data provide a comprehensive and recurrent overview of the total sales of antimicrobials in Europe. They confirm the huge differences between countries with the highest and lowest sales ranging from 2.3 mg/PCU to 393.9 mg/PCU and a median value of 51.9 mg/PCU. For the 25 countries which provided sales data for all years between 2011 and 2020, an overall decline in sales (mg/PCU) of 43.2% was observed, with a noticeable decrease in sales identified for some of the highest-scoring countries.

Although these ESVAC data are successful in monitoring the overall trends in AMU in animals in Europe, it is difficult to extract the specific evolution in AMU in pig production from this data. This is due to the fact that there are many antimicrobial compounds on the market that are registered for multiple species; therefore, these compounds cannot easily be assigned to one specific animal species. Nonetheless, in several European countries pig production accounts for a major part of the animal production and it can therefore safely be concluded that the observed reductions in AMU are certainly also partially due to reductions of AMU in pig production. Most likely associated with higher biosecurity measures and improved husbandry and farm health management practices (Postma et al., 2016; Laanen et al., 2014).

Herd level monitoring of use data

When moving from sales data to use data, typically herd level data collection systems are needed. In Europe, several countries have already established or are developing systems for monitoring AMU in pigs. Currently, the AACTING project describes 25 different herd level AMU data collection systems for pig production originating from 15 different European countries (AACTING, 2022; https://aacting.org/monitoring-systems/pig--data-collection-systems/?lid=1679). These systems differ in many ways, including the type of collected data, the performed analyses and their respective output. At the same time, they share key components such as data collection, analysis, benchmarking and reporting. As a result, they face similar challenges for which they need to make the appropriate decision (Sanders and Vanderhaeghen et al., 2020). For example, many herd level data collection systems are used in the framework of a benchmarking scheme to improve (and reduce) AMU and to promote antimicrobial stewardship actions. Further harmonization of methods and processes, as called upon by Sanders and Vanderhaeghen (2020), could lead to an improved comparability of outcomes and less confusion when interpreting results across systems. Having these type of animal specific data collection systems at farm level is extremely helpful as they allow to describe species-specific evolutions in AMU and assist in creating antimicrobial reduction goals over time. As an example, in Belgium, farm level AMU data of the pig sector is being collected since 2014. In agreement with the national sales data, AMU in the pig sector has quite steadily been decreasing over the years. For example, between 2018 and 2020, the data shows a decrease of approximately 10% in the sector-level treatment incidence. Interestingly, in 2020 the decrease appeared to have been solely situated in finisher pigs, which represent the largest mass of 'treatable animals'

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whereas in breeding pigs and piglets a steady state or slight increase was observed. This illustrates the usefulness of farm-level data to gain detailed insight in the situation in the field, allowing for focused and specific approaches.

Antimicrobial use in pig production in Europe: the way forward

Better health management and biosecurity

While the principal role of AMU in food animals should be therapeutic, in reality use has been substantially driven by the objective of improving farm productivity and income. High AMU has often been used as a tool to compensatefor poor health management on the farm. However, these are not acceptable practices. Therefore, husbandry systems, production systems and both management and biosecurity standards should be designed in such a way that the need for antimicrobials becomes exceptional or even redundant.

In a study by Postma et al. (2015), European veterinarians active in pig production were asked what they consider to be the most valid alternatives for AMU in pig production, taking into account expected effectiveness, feasibility and return on investment of the measures. Results indicated that practitioners believe the most promising alternatives to AMU are, in order of priority: improved biosecurity, increased and improved vaccination, use of zinc (against *E. coli* infections in weaned pigs), improved feed quality and improved diagnostics. Besides the use of zinc, which is banned for medicinal use in Europe since the end of 2021 (EC 2017), all the described alternatives are within reach for all pig producers.

In the meantime, several studies have found that improved biosecurity may result in reduced AMU, without jeopardizing production results. In a study in farrow-to-finish pig herds in Belgium, it was found that herds with higher internal biosecurity scores, determined by means of the Biocheck. UGent scoring system, had lower antimicrobial treatment incidences, suggesting that improved biosecurity might help in reducing the amount of antimicrobials used (Laanen et al., 2013). In a French study in farrow-to-finish herds, biosecurity measures such as disinfection of the loading area, gilt quarantine and adaptation, farm structure/working lines and all-in/all-out practices were found to be significantly associated with lower AMU (Lannou et al., 2012). In a multi-country study comprising four European countries, it was shown that a higher weaning age (>24 days), a batch management system of five weeks or more and the external biosecurity level were significantly associated with a lower antimicrobial treatment incidence (Postma et al., 2016). This finding was confirmed in a study of the characteristics of top pig farmers. In this study, the level of internal biosecurity was positively associated with a better control of infectious diseases and a lower need for antimicrobials (Collineau et al., 2017a). In Denmark, farmers and their veterinarians implemented measures that managed to reduce their annual antimicrobial consumption by 10% or more following the introduction of the "Yellow Card system". It was reported, among other parameters, that cleaning procedures, adequate action regarding diseased animals (e.g. an earlier decision to euthanize) and all-in/all-out systems were mentioned by farmers and veterinarians as means to reduce AMU (Dupont et al., 2017). Another study concluded that improved biosecurity, especially the presence of a hygiene lock, and pest control by a professional company, were related to lower probabilities of farms being infected with extended spectrum beta-lactamase producing *E. coli* (Dohmen et al., 2017). An intervention study in Belgium found that improving pig herd management and biosecurity status, in combination with antimicrobial stewardship, helped reduce AMU in pigs from birth till slaughter by 52%, and in sows by 32% (Postma et al., 2017). In the latter study, the management and biosecurity interventions were generally relatively simple for farmers to implement. They included changing the working habits and routines of the farmer (e.g. changing of needles, hand and personal hygiene, and analysis of water quality). Interventions incurring higher costs and/or requiring more pronounced changes, such as introducing a new hygiene lock to change clothes/boots and wash hands, were implemented less frequently. A key recommendation was having a good and early registration of disease signs allowing to take proper and timely control measures (e.g. biosecurity, vaccination and climate change), and to create awareness of the importance of the principle that "prevention is better than cure". An economic evaluation based on the results of this study has shown that, including labour costs of all persons involved (including the coach, veterinarian and farmer), the participating herds achieved an average financial gain or overall benefit of €2.67 per finisher pig per year from partaking in this "team effort" approach (Rojo-Gimeno and Postma et al., 2016). In a comparable study performed in four European Union countries, an economic evaluation of suggested interventions in, among others, biosecurity resulted in a median change in net farm profits among Belgian and French farms estimated at €4.46 and €1.23 per sow per year, respectively (Collineau et al., 2017).

Towards zero antimicrobial use

Raised Without Antibiotics (RWA) is a certification mark that is known in countries such as Denmark and the United States. However, specific inclusion criteria for RWA production and the implementation of RWA in a large number of herds with varying management and housing conditions has only been limitedly investigated. In a recent Belgian study (not yet published) twenty-eight Belgian pig herds were enrolled and their AMU as followed for a period of 35

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months. The goal of the study was to evaluate to what extend pig farms could be coached towards antimicrobial free pig production and to what extend they could also maintain this status over time. In this study RWA was defined as no antibiotics from birth until slaughter. Pigs requiring an individual treatment received a special ear-tag and were excluded from the RWA program. The results of the study showed that 13 out of the 28 herds were successfully raising pigs without antibiotics after a coaching period of one year. One year later still 12 out of the 13 were maintaining this status. Remarkably RWA herds applied less vaccinations, were smaller (median 200 sows, range 85 – 300 compared to non-RWA herds median 350 sows, range 180 - 1250), applied more frequently a 3- and 5-week batch farrowing system, compared to the 4-week system which was used significantly more in non-RWA herds. The weaning age was slightly higher (not significantly) on RWA farms (mean 24.9 days) compared to non-RWA farms (mean 23.9 days). This study showed it was possible for farmers to achieve and maintain the RWA status through herd-specific coaching related to prudent AMU and biosecurity.

Conclusions

Based upon the described evolution in AMU in pig production in Europe in the last decade and based upon the results already today obtained by the leading producers and countries, it is clear that the use of antimicrobials will further diminish and ultimately will become an exceptional act in future pig farming. Obviously, for the majority of the farms both within and beyond Europe this will require further efforts and focus on better husbandry, biosecurity and management. Ultimately this reduction will also result in the levelling off, and eventually even reversal, of resistance selection, leading to further benefits for animal health as well as human health, global food safety and food security.

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Antimicrobial use in pigs in North America

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Introduction

The goal herein is to describe antimicrobial use in North America and to survey the influences on use with focus on the rapidly changing regulatory environment. It is critical to recognize a few realities in North America that impact the accuracy and completeness of the following paper and warn the reader to be aware. First, most available studies are survey based with the standard biases that are recognized in that form of assessment. Secondly, antimicrobial use and motivations for use varies at the farm level and are often viewed as proprietary information (or at least at risk of impacting marketing opportunity) which creates some reluctance to share information. Third, the final form of many regulatory rules, especially in the antimicrobial arena, are influenced not only by the scientific evidence but also the local, state, and regional norms and values of the population at large including consumers and non-consumers of the end product that may have no technical understanding of resistance or antimicrobial function. Farmers, producers and veterinarians are seldom the majority voices in these populations. Fourth, most legislative bodies (especially those in the United States) were in session at the time this proceeding was due. Potential legislation at the time of the writing of these proceedings many have passed into law, been abandoned, or changed significantly. Fifth, regulation of antimicrobial use in all areas of medicine, both human and animal, is occurring at not only federal and state/provincial levels but also locally in cities, parishes, districts, and counties. Finally, there is no single, comprehensive database or tracking mechanism for antimicrobial use or pending legislation in any of the North American countries. Veterinarians need to find a reliable source for the jurisdictions that affect their clients and stay engaged in the monitoring. It is worth noting that both antimicrobial use (or surrogates of use, like sales data) and the prevalence of resistance (primarily detection of genes with PCR or sequencing) are tracked together and separately. This hinders the establishment of meaningful correlations between use and specific resistance outcomes. These proceedings will describe general trends and more specific information on the 23 sovereign states of North America will be presented at the meeting.

Why does concern about antimicrobial use and resistance seem to be intensifying?

The short answer is the true magnitude of the problem seems to continue to grow and, in part is better understood today than in the past. Antimicrobial resistance (AMR) is a local problem (individual animal?) when bacteria have or develop the ability to defeat the drugs used to kill them. AMR is a global problem when that resistance becomes mobile, outlives the host, and is packaged in a way that allows it to move globally. One of the key mechanisms, the accumulation of genes conferring resistance into plasmids that can be transferred among bacteria, also allows for selection pressure from one antimicrobial to concurrently select for resistance against other antimicrobial options. This multi-drug resistance magnifies the consequence of selection pressure due to drug exposure and also globalizes resistance. While the target bacteria of an isolated treatment decision may not travel the globe as a pandemic, their resistance genes can and do. Thus, it is established that antimicrobial use in one setting likely impacts antimicrobial efficacy in other settings. It is time to transition our paradigm of antimicrobials as reliable, inexpensive tools that are renewable to a perspective that antimicrobials are a non-renewable resource of last resort when animal health and welfare are threatened.

Antibiotic-resistance bacteria, according to the Centers for Disease Control, cause at least 2 million illnesses and 23,000 deaths in the United States every year. In fact, a recent comprehensive analysis of the impact of AMR found that "by 2050, 10 million lives a year and a cumulative 100 trillion USD of economic output are at risk due to the rise of drug-resistant infections if we do not find proactive solutions now to slow down the rise of drug resistance" (from Tackling Drug-Resistant Infections Globally: Final Report and Recommendations of the Review on Antimicrobial Resistance (available at amr-review.org). AMR is truly an ecosystem problem, impacting human, animal, and environmental health, and for this reason addressing the problem requires multidisciplinary teamwork and research designs. This growing public health threat has prompted action on a number of fronts and one way is increasing regulatory oversight for antimicrobials used in food producing animals including swine.

Basic concepts of antimicrobial resistance

To understand why antimicrobial use in livestock is targeted for public and regulatory concern, a basic understanding

and common reference language is useful. Intrinsic AMR is due to the typical structure or function of a bacteria rather than an acquired modification. For example, the bacteria may simply not have the target structure or enzyme typically bound by the antimicrobial. This is the best explanation for class differences in efficacy among bacteria. Gram-negative bacteria are intrinsically resistant to some antibiotics because the antibiotics cannot cross the cell wall to enter the bacteria. Beta-lactams' ineffectiveness against mycoplasma species is another example. There is a wide variety of mechanisms that provide bacteria intrinsic resistance to antimicrobials. Understanding these mechanisms is useful for developing new antibiotics. Both intrinsic and acquired mechanisms are genetically coded and can arise or disappear with genetic mutation of the bacteria. Often discussions of a bacteria's "spectrum" reflects which bacterial species have intrinsic resistance and which ones do not. Most bacteria have intrinsic resistance to some antibiotics. Generally, intrinsic resistance mechanisms are transferred vertically from bacteria parents to bacteria offspring.

Acquired resistance is due to mechanisms that are more readily transferred between bacteria often using lateral transfer mechanisms such as conjugation, transformation, and transduction that allow bacteria of the same generation to pass genes between themselves without replication. Conjugation is usually the transfer of a plasmid containing one or more genes when two bacteria are in contact. Transformation is the ability of bacteria to pick up genes directly from the environment and use them. Transduction is the transfer of genetic material between two bacteria by a virus or phage. Important to the spread of acquired AMR is the ability of these mechanisms to facilitate transfer between bacteria of different species.

Acquired resistance mechanisms fall into three broad categories: a) those that reduce the concentration of the antibiotic in the cell either by preventing penetration or rapidly pumping it back out of the cell, b) those that modify the target of the antibiotic so that it can't bind or affect the target and c) those that inactivate or modify the antibiotic itself, usually by hydrolysis.

It is important to understand that exposure to antibiotics promotes the development of both intrinsic and acquired resistance mechanisms. Because of the ability of bacteria to transfer acquired resistance mechanisms to bacteria of other species, all antibiotic exposure becomes relevant to human and animal health. This also means that all antimicrobial use presents opportunities to reduce the pressure for selection of resistance mechanisms by employing deliberate stewardship efforts. In swine production, we must consider that environmental exposure, treatment of healthy animals, prophylactic use and subtherapeutic use are situations where a sick animal does not benefit but a resistance mechanism might be selected or promoted. Further, the development of resistance is relevant to both human and animal health even when pathogenic bacteria are not exposed due to the ability of bacteria species to laterally share resistance genes.

There is growing evidence that AMR is impacting the successful treatment of swine cases and becomes immediately important to the swine practitioner independently of broader food and human safety issues. *Escherichia coli* with multi-drug resistance (MDR) and growing resistance to ceftiofur, enrofloxacin, florfenicol, gentamicin, neomycin and sulfonamides are a concern to practitioners. Other primary swine diseases that are being monitored closely for their increasing resistance include *Salmonella ssp*, *Streptococcus suis*, and *Pasteurella multocida*.

Antimicrobial use

Within the 23 sovereign states of North American, nearly all conceivable structures of swine production and housing, disease ecologies, degrees of industrialization, and structure of veterinary training exist. Further, nearly the entire range of climates are represented and the cultural roles of pigs and pork vary widely among regions and even within countries. Generally speaking, Canada, the United States and Mexico have the largest production systems and the most intensive regulation of antimicrobial use with both subtherapeutic use for growth promotion banned and the majority of therapeutic use requiring the authorization of a licensed veterinarian.

In the U.S., antimicrobial use and stewardship have been surveyed and reported by the Animal and Plant Health Inspection Service of the United States Department of Agriculture. The latest report, released in 2019, "…represents the Nation's first in-depth look at antimicrobial use and stewardship practices on U.S. swine sites. The study was designed to collect information about antimicrobial use and stewardship practices on U.S. swine sites from July 1 through December 31, 2016—before the U.S. Food and Drug Administration (FDA) implemented antimicrobial use policy changes on January 1, 2017. The FDA changes included eliminating the use of medically important antimicrobials for growth promotion purposes in food animals and requiring veterinary oversight for the use of medically important antimicrobials in animal feed or water." (https://www.aphis.usda.gov/animal_health/nahms/downloads/amu-swine-operations.pdf). The agency is currently

collecting data that would reflect the impact of those changes in subsequent years. Ninety percent of all market pig sites administered antimicrobials in feed or water or both and about one-third of those were described as being for

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growth promotion. While this does not reflect current practices, it will make a useful baseline for comparison.

A study from 2010 illustrates the challenges of global estimates. In this report, the United States was estimated to be one of the top 5 countries with the largest antimicrobial consumption in food animals with 13% of global use. When use in livestock was projected to 2030, Mexico is expected to join the top 5 with 2% of global use. The projections are likely already altered by the aforementioned regulatory changes in the United States that have occurred since. Importantly, the lack of consistent information among countries was noted as a challenge and led to the use of indirect means to estimate antimicrobial consumption in livestock (Van Boeckel, Brower, 2015).

The United States Food and Drug Administration reports annually on antimicrobials sold for use in food animals. The most recent report (https://www.fda.gov/animal-veterinary/cvm-updates/fda-releases-annual-summary-report-antimicrobials-sold-or-distributed-2019-use-food-producing) describes: "..domestic sales and distribution of medically important antimicrobials approved for use in food producing animals increased by three percent between 2018 and 2019. The trend over time indicates that ongoing efforts to support antimicrobial impact are having an impact: sales and distribution are down 25 percent since 2010 and down 36 percent since 2015, which was the peak year of sales."

The most granular data in the United States is from a report describing a pilot study to assess the feasibility of voluntarily sharing proprietary use data from production systems (Davies and Singer, 2020). In this study, approximately 60% of use (by weight) in market animals were drugs of the tetracycline class, which suggests perhaps a way to prioritize stewardship focus for the fastest impact on the surface. However, this varied enough between farms that the value of the data as actionable guidance is eroded as it is aggregated at higher levels. The effort to collect existing proprietary data emphasized and revealed a number of biases that have to be considered when establishing a national or international index but offered a valuable proof of concept.

Specific regulatory initiatives

Legislative initiatives to monitor or restrict antimicrobial use in food animals are in various states of development in several states. Senate Bill 27 in California was approved in 2015 and, among other provisions, "require(s) the Department of Food and Agriculture, in consultation with the Veterinary Medical Board, the State Department of Public Health, universities, and cooperative extensions, to develop antimicrobial stewardship guidelines and best management practices on the proper use of medically important antimicrobial drugs and would require the department to gather information on medically important antimicrobial drug sales and usage, antimicrobial resistant bacteria, and livestock management practice data."

Legislation to prohibit antimicrobial use in food-producing animals, particularly prevention uses, and to require reporting by veterinarians was enacted in Maryland (HB 652/SB 471). The bill requires veterinarians to provide information on antimicrobial use, especially those deemed "medically important antimicrobial drugs." The Maryland legislation defines these as: "…means any drug from a class of drug or derivative of a class of drug that is:
(i) Made from a mold or bacterium that kills or slows the growth of other microbes, specifically bacteria; and
(ii) Used in human beings or intended for use in human beings to treat or prevent disease or infection;
OR is listed in either:
(i) Appendix A of the federal Food and Drug Administration's Guidance for Industry #152, including critically important, highly important, or important antimicrobial drugs
or
(ii) a subsequent guidance document created by the federal Food and Drug Administration that ranks the medical importance of antimicrobial drugs."

The reference to the federal lists is important to recognize as the current federal list and industry guidance is under review and a draft has been proposed for public comment. Also notable in the Maryland legislation was the implementation of a limit of administration to 21 days unless the federally approved label required a longer duration of therapy. Further, swine farms selling fewer than 200 animals per year were excluded from the provisions of the law.

A similar bill was introduced in New York (S. 5742) but failed to pass before the session ended. Of interest, that bill referred to the World Health Organization (WHO) list of important, highly important and critically important drugs when defining which ones would be the focus of the legislation rather than the FDA guidance document. The New York bill added "in relation to surgical or other medical procedures" as a condition that qualified for veterinary ordered use of medically important antimicrobials. It also required a farm visit within the previous six months by the veterinarian before prescribing.

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The Illinois State Senate also considered a bill with restrictions on use of antimicrobials in food-producing animals (SB 1186) but failed to pass it before the session ended. Bills in Pennsylvania and Oregon are also being drafted. These individual state actions have the potential to create a confusing web of requirements and definitions for multistate practitioners.

Selected responses to the problem and relevance to swine medicine practice

Initiated in 2018, The National Institute of Antimicrobial Resistance Research and Education's (NIAMRRE) mission is to drive collaborative and integrative research, education and engagement to solve AMR challenges and benefit society using a One Health approach. NIAMRRE will also provide local, national, and international leadership in combating antimicrobial resistance; generating evidence-based solutions for antimicrobial stewardship; contributing to improvements in the health of animals, humans, and the environment (One Health); and facilitating economically and socially sound policy development and implementation. In short, NIAMRRE will foster an active understanding of AMR to reduce its societal impact. This will be achieved through research, education, collaboration, and advocacy efforts. In September 2014, The White House released the National Strategy on Combating Antibiotic Resistant Bacteria (CARB). In response, the Association of Public and Land Grant Universities (APLU) and the American Association of Veterinary Medical Colleges (AAVMC) launched a joint Task Force on AMR in Production Agriculture. In September 2015, The White House established a federal interagency Task Force called the Presidential Advisory Council on Combating Antibiotic-Resistant Bacteria (PACCARB). Concurrently, APLU and AAVMC released a report offering an array of research and education recommendations designed to address the problem utilizing a One Health approach. The report called for the creation of a National Institute of Antimicrobial Resistance Research and Education (NIAMRRE) to coordinate the implementation of the report's recommendations. In April 2018, The APLU and AAVMC requested proposals to host the institute in a manner that incorporates the vision, mission, direction and principles contained in a framing concept paper. After a national search for host institutions and evaluation of nine high quality proposals in July 2018, a pre-existing regional Antimicrobial Resistance Consortium based at Iowa State University was selected to develop NIAMRRE. Key players in the early consortium included the University of Iowa, the United States Department of Agriculture National Center for Animal Health, University of Nebraska-Lincoln, the University of Nebraska Medical Center at Omaha, and Mayo Medical Clinic in Rochester, MN. In January of this year, NIAMRRE core staff were hired and work began to determine first-year priorities, governance structure, and a potential membership model.

The Presidential Advisory Council on Combating Antibiotic-Resistant Bacteria (PACCARB) was established in September 2015 by Executive Order and was recently codified in the Pandemic and All Hazards Preparedness and Advancing Innovation Act of (PAHPAIA) 2019. The mission is to provide the Secretary of Health and Human Services with advice, information, and recommendations on policies and programs related to antibiotic resistance from a One Health perspective. The membership consists of globally recognized experts from both human and animal domains within academia, the biomedical and pharmaceutical industries, public health, and advocacy organizations. Public meetings explore every topic area through human, animal, and as appropriate, environmental domains, from both domestic and international perspectives to inform reports that are drafted to include both human and agricultural priorities for the U.S. Government's attention. In their latest 2019 report, the PACCARB prioritized the need for continued research, development, and implementation of best practice interventions for antibiotic stewardship and infection prevention within AND across all human and animal healthcare settings through evidence-based approaches. Between 2016 – 2019 PACCARB has produced four Recommendations Reports: Initial Assessments of the National Action Plan for Combatting Antibiotic-Resistant Bacteria (2016), Recommendations for Incentivizing the Development of Vaccines, Diagnostics, and Therapeutics to Combat Antibiotic Resistance (2017), Key Strategies to Enhance Infection Prevention and Antibiotic Stewardship: Report with Recommendations for Human and Animal Health (2018), and Priorities for the National Action Plan on Combating Antibiotic-Resistant Bacteria: 2020 – 2025 (2019)

PACCARB voting and liaison membership includes a swine veterinarian, a beef veterinarian and several commodity groups as well as medical doctors, clinicians, researchers and nurses. All archived meeting summaries, videos, presentations, and council reports are available at: www.hhs.gov/oash/carb | Email: CARB@hhs.gov.

PACCARB has expressed support for federal efforts to improve antimicrobial development and address the failing new antimicrobial pipeline which include two bills being considered at the time of these proceedings (excerpt from Report 10 – Recommendation on proposed legislation (https://www.hhs.gov/sites/default/files/paccarb-incentives-leg-letter-oct-6-2021.pdf):

> "Two bills introduced into Congress seek to address these root causes—the Developing an Innovative Strategy for Antimicrobial Resistant Microorganisms (DISARM) Act and the Pioneering Antimicrobial Subscriptions to End Upsurging Resistance (PASTEUR) Act. Both of these bills are supported by industry, physicians, pharmacists, and

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patient advocacy organizations as demonstrated by endorsements from the Biotechnology Innovation Organization, Infectious Diseases Society of America, and others.

The PACCARB strongly supports the passage of both DISARM and PASTEUR and the antimicrobial stewardship provisions within. The DISARM Act would modify the hospital payment system to incentivize the use of newer, more expensive antimicrobial drugs in those patient encounters that are appropriate. In the diagnosis-related group (DRG) payment system, hospitals receive a flat payment for treatment of a condition regardless of the costs incurred, leading to selection of the least expensive treatment options. DISARM would provide payment outside of the DRG for use of certain antimicrobial drugs, providing hospitals with the financial freedom to select newer drugs when appropriate, and thereby enabling appropriate patient access. To complement this approach, the PASTEUR Act would remedy the reduced revenue realized as a result of appropriate and much needed antibiotic stewardship efforts by creating a subscription payment model for new antimicrobial drugs. Under this model, the U.S. Government enters into a purchasing contract that delinks payment from sales volumes, thereby significantly reducing the risk to companies wishing to develop new antimicrobials that fulfill critical needs while still ensuring appropriate use that preserves these new agents for the future. Crucially, the minimum contract size ($750 million) is sufficiently large to provide a real incentive to change research investment decisions."

A number of government and professional organizations are funding research into reporting mechanisms. It is accepted that combating antimicrobial resistance requires judicious use of antibiotics. Judicious use of antibiotics is contingent on correct identification of therapeutic opportunities using diagnostic resources with effective communication between the case veterinarian and the laboratory diagnostician. Veterinary test results require useful context for interpretation. While this is accepted for many clinical parameters (such as the normal ranges that accompany blood cell panels or mycotoxin concentrations) it appears that most antibiotic susceptibility reporting is structured for efficiency, by legacy formatting, reflects the formatting of software systems/databases or is a consensus structure that is expected to achieve utility for multiple species. Concurrently, there have been significant efforts, supported by the AASV and others, to improve the sharing of data between labs, summarize antibiotic susceptibility trends within and among farms and laboratories, improve the ability to manipulate this data in real time, and both access and aggregate case diagnostic results on a variety of electronic platforms. Anecdotally, there are significant differences in the form and content of susceptibility data that veterinary and human laboratories report. We were unable to locate evidence of systematic evaluation of whether knowledge of testing procedures or the format of culture and susceptibility reports from veterinary diagnostic laboratories (VDLs) influence antimicrobial selection decisions.

The highest priority needs and recommended approaches to address them have been defined by both the National Action Plan for Combating Antimicrobial Resistant Bacteria and the report from the Joint APLU | AAVMC Task Force on Antibiotic Resistance in Production Agriculture. The Task Force made two recommendations, "(1) design and implement a model curriculum to improve awareness, understanding and help in the implementation of effective actions to combat antibiotic resistance, and (2) develop and implement educational and informational strategies, tools and programs that focus on different groups extending across our education spectrum. The Antimicrobial Resistance Core Competencies Working Group was formed to further those two Recommendations." The Working Group included expertise in teaching and in antibiotic resistance and produced a framework of 60 desired learning outcomes for veterinary professional curricula. Clearly these efforts collectively seek to change (or improve from the outset) veterinarian behavior towards more judicious use of antimicrobials. Consistent with these efforts, AASFV has funded research to test the strategies of providing detailed training on the process that produces susceptibility case data and expanding the context provided with the results to change veterinarian and veterinary student behavior at the case level.

In September 2018, the U.S. Food and Drug Administration Center for Veterinary Medicine published "Supporting antimicrobial stewardship in veterinary settings, goals for fiscal years 2019 – 2023" which outlined several goals (with objectives and actions) relevant to swine medicine practice. Those goals, objectives and actions with direct relevance to swine practice are listed below. (The entire document, including objectives and actions for companion animals can be found here: https://www.fda.gov/files/animal%20&%20veterinary/published/Supporting-Antimicrobial-Stewardship-in-Veterinary-Settings--Goals-for-Fiscal-Years-2019-2023.pdf)

GOAL 1: Align antimicrobial drug product use with the principles of antimicrobial stewardship

Objective 1.1: Revise, as necessary, the use conditions for approved medically important antimicrobials in food-producing animals

Action 1.1.1: Publish a list of medically important antimicrobial drugs administered in the feed or drinking water of food-producing animals that are approved for indications that lack a defined duration of use.

Action 1.1.2: Issue a draft strategy (e.g. GFI) to ensure that all medically important antimicrobial drugs used in the feed or drinking water of food-producing animals have an appropriately targeted duration of use.

Action 1.1.3: Issue a draft strategy (e.g. GFI) to bring all dosage forms (including, injectable, intramammary, etc.) of medically important antimicrobial drugs approved for use in food- producing animals under the oversight of a licensed veterinarian.

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Action 1.1.4: Issue and implement a final strategy8 (e.g. GFI) to bring all dosage forms (including, injectable, intramammary, etc.) of medically important antimicrobial drugs approved for use in food-producing animals under the oversight of a licensed veterinarian.

Action 1.1.5: Engage with stakeholders on how antimicrobial product label information could better support antimicrobial stewardship.

Action 1.1.6: Issue a final strategy (e.g. GFI) to ensure that all medically important antimicrobial drugs used in the feed or drinking water of food-producing animals have an appropriately targeted duration of use.

Objective 1.2: Develop and implement a strategy for promoting antimicrobial stewardship in companion animals

Objective 1.3: Enhance processes to support new product development

Action 1.3.1: Publish a draft of revised Appendix A of Guidance for Industry #152 to update the list of medically important antimicrobials.

GOAL 2: Foster stewardship of antimicrobials in veterinary settings

Objective 2.1: Support outreach and education by providing information on antimicrobial stewardship

Objective 2.2: Strengthen CVM compliance program activities to support antimicrobial stewardship.

Action 2.2.2: Expand the comprehensive VFD compliance strategy to integrate the VFD inspection framework into inspections associated with the Drug Residue Inspection Program.

Action 2.2.3: Initiate steps to identify and address the inappropriate marketing of antimicrobial drugs. (e.g., illegal marketing of unapproved animal drugs containing medically important antimicrobials)

Objective 2.3: Support international outreach and collaboration to foster antimicrobial stewardship in veterinary settings

Action 2.3.1: Collaborate with other federal agencies to develop U.S. Government positions and engage international partners on activities to combat antimicrobial resistance. This includes engaging other developed countries and organizations like the World Organization for Animal Health (OIE), World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO), Codex Alimentarius, and the Transatlantic Task Force on Antimicrobial Resistance.

GOAL 3: Enhance monitoring of antimicrobial resistance and antimicrobial drug use in animals

Objective 3.1: Collect and analyze data on antimicrobial drug use in animals

Action 3.1.1: Complete pilot projects initiated in 2016 to characterize antimicrobial use practices in the fourmajor food animal species (cattle, swine, chickens, and turkeys).

Action 3.1.2: Finalize an appropriate method for applying a denominator to available antimicrobial sales and distribution data.

Action 3.1.3: Develop a long-term strategy for implementing a functional and efficient antimicrobial use monitoring and reporting system for veterinary settings.

Objective 3.2: Enhance the collection and analysis of antimicrobial resistance data

Action 3.2.4: Build and increase domestic capacity to monitor antimicrobial resistance in animal and zoonotic pathogens to include companion animals and animal feed.

Objective 3.3: Increase data sharing and reporting to aid in the monitoring of antimicrobial drug use practices and resistance

Action 3.3.4: Publish a report of the information gathered through CVM-funded cooperative agreements that characterize antimicrobial use practices in the four major food animal species (cattle, swine, chickens, and turkeys)

Programs as precedent to legislation and regulation

There is a financial incentive to implement formal stewardship programs, including comprehensive training programs, in human medicine and there are a variety of programs that are now developed, implemented, and accruing outcomes information. In the absence of a direct financial incentive to adopt stewardship programs in veterinary medicine, we run the risk of inaction while programs in the human medicine space mature. Once a program or process emerges there with clear evidence of impact on antibiotic resistance, it will be tempting to regulate or legislate its adoption in food animal medicine. Leveraging a program developed in a human medicine practice space into a food animal practice space is fraught with complication and once a regulatory approach is employed to do it, the animal level work to comply will fall to farmers, veterinarians, and producers. The pork production industry and swine veterinarians would be better served to fund, develop, validate and train our own programs that succeed in the unique practice space where we operate.

Summary

We have only recently begun to understand the expansive scope, speed and complexity of AMR and accepted the reality that significant future risks to humans and animals are rapidly developing and re-emerging. The question at

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this time is whether we can learn the mechanisms, adapt our behaviors and improve antimicrobial stewardship in time

to preserve the utility that remains. Legislation establishing regulation is in progress at local, state, national levels. The details of bills, passed and proposed, at the state level has the potential to create a complex web of varied requirements that will further challenge interstate practice and national production systems. Successful programs that accumulate evidence of behavioral change by prescribers in human health professions will likely be seen as potential "solutions" that could be extended to swine medicine via legislation. Swine veterinarians would be better served to embrace and document the core concepts of formal stewardship plans, much of which they are already doing.

References available upon request.

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Antimicrobial use in pigs in Brazil

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Introduction

Brazil is the 4th largest producer and exporter of pork in the world and the second largest consumer of antibiotics for food-producing animals (Tiseo et al., 2020). The countries' economy is highly dependent on the export of its agricultural products (von Keyserlingk and Hötzel, 2015). The importance of the country in world meat production requires that the Brazilian pig industry attends to the demands of the modern consumer market, especially in relation to food safety, animal welfare and environmental sustainability. Concerns with AMR and antibiotic residues in the products and the environment fall within this scope. Scientific evidence identifies the use of antibiotics in intensive livestock farming as a risk factor for the occurrence of antimicrobial resistance (AMR) in animals and humans, which may force the Brazilian pig industry to change its relationship with antibiotics.

The international call by important world health agencies (FAO, OIE and WHO) to combat the AMR problem seeks to establish programs to monitor and foster the rational use of antibiotics. Moreover, develop and enact recommendations of active principles considered of clinical importance for human health (Organization, 2016). As a signatory member of the OIE, Brazil has undertaken to establish guidelines for the use of antibiotics in food-producing animals through the PAN-BR AGRO Program (Brasil, 2019). PAN-BR Agro seeks to increase the awareness of veterinarians, agricultural technicians and rural producers about the rational use of antibiotics, besides monitoring resistant bacteria strains, promoting legislative changes in the use of veterinary antibiotics, among other actions.

The antibiotic use /resistance challenge is often considered a health problem to be solved by increasing awareness; yet, building evidence indicates that AMR is accelerated by social, cultural and economic structures that lead to the misuse, overuse and abuse of these life-saving medicines (Minssen et al., 2020). For this reason, it is essential to know the attitudes, rationale and drivers around antibiotic use practices of those involved in the pig production chain, including farmers and all other professionals/roles that make up the pig industry. This knowledge can help to understand the preparedness/willingness to make structural changes aiding in building a holistic strategy that better engage all stakeholders needed to build a change of structures that facilitates a rational antibiotic use.

Antibiotic use in Brazilian pig farms

Similar as in other major pig producing countries, Brazil uses antibiotics in three ways: as growth-promoting additives, for treatment of infections and as prophylactics in situations where pigs are expected to be stressed and vulnerable to environmental pathogens. Brazil has already limited many active principles that can be used for growth promoters (MAPA, 2020). Thus, the main use of antibiotics in pigs occurs for disease prevention, yetthe legislation does not provide for regulations regarding this form of use. Moreover, many antibiotics that are banned for growth promotion are being used for prophylactic purposes during the same periods they would be used for growth promotion (Albernaz-Gonçalves et al., 2021a).

Regarding the prophylactic use of antibiotics, the weaning phase is an example in which use of antibiotics is done continuously or in "shocks" (i.e., strategic periodic metaphylactic treatment), in order to avoid enteric and respiratory infections in weaned piglets (Albernaz-Gonçalves et al., 2021a). Several scientific studies identify this phase as one of the most important in relation to the vulnerability of pigs to infectious diseases (van Rennings et al., 2015). A quantitative survey of AMU in Brazilian pig farms (Dutra et al., 2021) indicate that pigs receive antibiotics for 73 % of their life, an information that is supported by farmers' reports (Albernaz-Gonçalves et al., 2021a). Brazilian studies cite aminopenicilins (amoxicillin) as the main antibiotic used in pig herds, as well as the use of tetracyclines (doxycycline), pleuromutilins (tiamulin), amphenicols (florfenicol) and polymyxins (colistin) (Albernaz-Gonçalves , 2012, Dutra 2021), meaning that some molecules classified by the World Health Organization as critically important antimicrobials for human medicine (WHO, 2019)are widely used in pig production in Brazil.

Several factors contribute to the high use of antibiotics in pigs. Management focused on adopting biosecurity practices can lead to successful reduction of use of antibiotics in pig farms (Rojo-Gimeno et al., 2016; Raasch et al., 2020).

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However, the biosecurity conditions of Brazilian pig farms, as well as the structures of the farms themselves, are an important reason behind the use of antibiotics. For example, a study conducted with pig farmers in the state of Santa Catarina identified that practices such as water chlorination and quarantine are adopted by less than 30% of the farms visited (Albernaz-Gonçalves et al., 2021a). Although this was a qualitative study on 58 farms, regardless of whether or not they were linked to agro-industries, farmers acknowledged that they could improve their hygiene and biosecurity standards and that this could reduce the need for antibiotics; however, they did not feel motivated to adopt such simple practices.

Additionally, several management factors used in Brazilian farms negatively impacts the immunocompetence of pigs facilitating the occurrence of infections and thus exacerbating the use of antibiotics (Albernaz-Gonçalves et al., 2022). Among them, management practices such as castration, tail clipping, teeth clipping cause pain and stress in piglets and can be risk factors for infections such as neonatal diarrhea. Moreover, common, yet negative animal welfare practices such as cross-fostering, early weaning, repeated social mixtures and transportation to new sites, are other powerful stressors that act as important risk factors for continued and high use of antibiotics in the growing phase. Other sources of stress in the growing phase as well as breeding pigs are barren environments (e.g., housing without environmental enrichment), high stocking density and spaces that limit movement. Besides their known negative effect on the welfare of pigs (Albernaz-Gonçalves et al., 2022), these practices are associated with high use of antibiotics (e.g., (van Rennings et al., 2015; Stygar et al., 2020; Lynegaard et al., 2021). Most of these practices (e.g.,use of environmental enrichment, group gestation housing, mandatory control of pain) have been regulated and shouldimprove in Brazil in coming years (Brasil, 2020). However, farmers have up to 25 years to implement changes.

Social factors that strengthen the use of antibiotics

Pig production has important economic and social importance in many regions in Brazil. Although most of the pig production is concentrated in the Southern region (in the states of Rio Grande do Sul, Santa Catarina and Paraná), other regions present relevant performances, such as the southeastern and mid-western regions. Unlike chicken production, in which there is a predominance of integration systems with large agroindustries, Brazilian pig farming ranges from farms with large herds and high technology to small scale, independent producers with exclusively family labor, that supply the local market. Depending on the region of the country, there is a predominance of independent producers with full-cycle farms, specialized farms linked to integration systems or agro-industrial cooperatives that supply mainly export markets. This heterogeneity of the chain not only defines different market sectors they supply to – e.g., external/international or the internal/national markets – but also involves important structural differences, in management, access to veterinary services/medicines and the type and frequency of technical assistance received by these producers.

However, even considering these differences between integrated and independent farmers, the excessive use of antibiotics is present in all production models. This is highly based on the reported (REF) confidence in antibiotics, framed on a belief in the effectiveness of these drugs as guarantors of health and profitability. This culture is well ingrained in the different links of the production chain. The verticality of the Brazilian pig chain allows a homogenization of practices among pig farmers bounded to determinations of the agroindustries. In integrated models, the rural producer receives the specialized information and technical assistance of the integrator, and assumes responsibility for the labor and the infrastructure of the farm; the companies supply the animals, feed and medicines; the farmers receive, together with the package of medicines, a list with recommendations for the responsible use according to the age of the animals, such as dosage, route of administration, withdrawal period. This allows producers to identify sick animals and treat them as recommended in the list. In more serious or difficult cases, agricultural technicians or and veterinarians are called in to provide technical assistance. Although there is a guide of agroindustries in relation to the types and amounts of antibiotics used based on symptoms, the free access to these drugs facilitates farmers to use them at will even if this use is inappropriate, e.g. using antibiotics for viral infections, or in inadequate doses (Albernaz-Gonçalves et al., 2021a).

The legislation requires antibiotics' sales to be signed off by a veterinarian, but there are many practical loopholes that override this principle, thus antibiotics for rations are easily purchased without the need for a veterinary prescription, from vendors, agriculture houses and on the Internet. One of the actions of PAN-BR agro refers to the extra-label recommendations and the control of the purchase and sale of veterinary antibiotics in the country. Legislation restricting the purchase of antibiotics would hit independent producers more directly, as they need to acquire their own inputs. One common form of access of these farmers to antibiotics is via commercial representativeswho visit the farms and who, in exchange for selling medicines and feed products, provide technical assistance, creating a relation of co-dependance.

Additionally, a link between agroindustry, pharmaceuticals and nutrition companies that generates direct and indirect jobs for professionals from different sectors reinforces an important relation of dependence on antibiotics in the pig

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production chain, and may be a barrier for change (Albernaz-Gonçalves, 2021). For example, the supply of medicines by integrators can be advantageous for farmers because agroindustries negotiate better drug prices than the producer would follow in retail. However, facilitated access, while strengthening a corporate network with strong economic ties between the pig industry and pharmaceutical laboratories, can also stimulate inappropriate use. Animal nutrition companies are another industry that is strengthened by these partnerships, through the sale of inputs to agroindustry. Thus, restrictive legislation on the use of antibiotics would inevitably affect these economic and social relations of employment and income of this web of actors that depends on the sale of these drugs as a source of income.

Moving forward

Some essential steps to meet the goal of reducing the use of antibiotics in practice in Brazilian farms are to invest in biosecurity, in more ergonomic infrastructures that bring more comfort to animals, as well as reduce the use of painful and several stressful housing and management practices. Regarding the link of antibiotic use and pig welfare, implementation of the new regulation on pig welfare (Brasil, 2020) is essential to support the adoption of rational use of antibiotics in Brazil. Recommendations such as group gestation housing, hospital pens, raising the age at weaning, are some examples of proven actions that would improve the welfare of pigs, their immunocompetence/susceptibility to disease and that will facilitate the reduction of antibiotic use. However, the transition time of farms until 2045 may negatively impact actions of rational use of antibiotics. Thus, it is important that the pig industry anticipates these changes voluntarily.

A cultural change in the shared understanding and vision of farm animal welfare may be necessary to the success of initiatives to reduce antibiotic use in pig farms. Currently the use of antibiotics is justified by some on the grounds that it protects the welfare of the pigs. Indeed, industry stakeholders define animal welfare in terms of biological function and good production performance (i.e. curing disease or limiting disease progression to achieve production goals), minimizing the relevance of naturalness and affective states (i.e. preventing disease by providing good AW) (Benard and de Cock Buning, 2013; Balzani and Hanlon, 2020; Albernaz-Gonçalves et al., 2021b). Although it is beyond discussion that animal's health is an essential dimension, the lack of recognition of animal welfare in all its dimensions and impacts makes it difficult to acknowledge that interventions focused in improving animal welfare may improve pigs' quality of life and make the industry less dependent on antibiotics (Albernaz-Gonçalves et al., 2022).

Several studies in low- and middle-income countries, including Brazil show a relationship of antibiotic dependence in pig farming (Dyar et al., 2019; Lekagul, 2019; Albernaz-Gonçalves et al., 2021a). Transitioning to a more prudent AMU requires individual behavioral changes; however, pig farmers, who are often seen as the ultimate stakeholders responsible for changing the current AMU, are not sufficiently autonomous to determine substantial changes to reduce antibiotic use. Many farmers report feeling powerless to think or act differently, quoting that economic constraints, production standards or technical advice do not leave room for change (Molnár and Fraser, 2020; Albernaz-Gonçalves et al., 2021b). External markets are pointed by farmers and other stakeholders as positive catalysts for change in the use of antibiotics in pig farming; local consumers are often dismissed by many as not interested or aware of the problem, despite evidence on the contrary e.g., (Hötzel et al., 2020).

A call for pig farmers to rationally reduce AMU may succeed or fail pending external support and structural changes in the network that currently support the use of antibiotics as a structural material for production, at local, national, and international levels. This means that individual behavior changes are not enough nor sustainable in the long run; the pig industry, consumers, and governments need to support investments needed for any changes.

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Antimicrobial use and stewardship in pig production in East and South East Asia – an update

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Introduction

East and South East Asia together holds the majority of the world's pigs (FAO, 2022) even if the region has been severely affected by the African Swine Fever epizootic the last years. China do hold the outstanding largest population of 406 M, but there are also other countries like Vietnam (22 M) and Myanmar (19 M) holding large populations in a global comparison. The region has had almost a consistent economic growth the last decades (World bank, 2022), except for the last years because of the Covid-19 pandemic. The economic growth has driven the demand for a more varied diet away from the dominance of just staple foods. In response, the pig sector is expanding and restructuring from small scale family farming to intensives large scale farms (Marius et al., 2015; Yang et al, 2019). However, there is a large variation between countries, in some there is still a dominance of small-scale family pig holdings. The intensive large scale pig farming in the region uses considerable amounts of antimicrobial agents (Tiseo, et al. 2020), sometimes for growth promotion and frequently on a regular basis for disease prevention (e.g.,Hallenberg et al. 2020). The frequent use of antimicrobials, mainly the subset antibiotics that are used against bacterial infections, has caused a widespread occurrence of antimicrobial resistance in the region (Criscuolo et al., 2021).

Here is an overview of the antimicrobial use in the region presented, then an up-date about the resistance situation in the pig sector, followed by a discussion on the recent regulatory measures taken about antimicrobial use in the livestock sector and finally some options for curbing the emergence of antimicrobial resistance in order to safe- guard the efficiency of antibiotics in the pig sector in the region.

About the antimicrobial use in in the region

To present solid data on the antimicrobial use in pigs around the world one must overcome several methodological challenges. In some countries or regions of the world there are actually systematically collected sales data (ESVAC, 2021) or even use-on-farm data available for livestock as a whole. But for most countries there are not. Another challenge is to identify how much of the total use is connected to the various domestic animal species, in this particular case the pig. Thus, most global data are based on estimates that are generated from sales data from countries with actual records. Regarding the share of antimicrobials by the pig sector, it has been estimated that in 2017 pigs do get 193 mg antimicrobials/ population correction unit (PCU), compared with 68 and 42 mg/PCU for chicken and cattle, respectively (Tiseo et al 2020). Thus, based on this estimate a large pig population will lead to a very high consumption in antimicrobial agents (see **Figure 1**). For instance, the official reportion on sales of antimicrobials for livestock in China 2017 was 41,967 tonnes (Chinese Ministry of Agriculture, 2017) and the use for animals in Vietnam has recently been estimated to 1,155 tonnes annually (Carrique-Mas et al., 2020).

Given that antibiotics are relative cheap and readily available in the region, they are an appealing alternative to good animal husbandry practices to prevent disease for instance at farrowing, suckling and weaning (Hallenberg et al., 2020: Lekagul et al., 2019). In several cases the antibiotics are used without proper guidance from competent veterinarians, thus missing out a professionally made diagnosis that risks a mis-match between pathogen and efficient medicine as well as an inadequate dosing (eg., Ström et al., 2018).
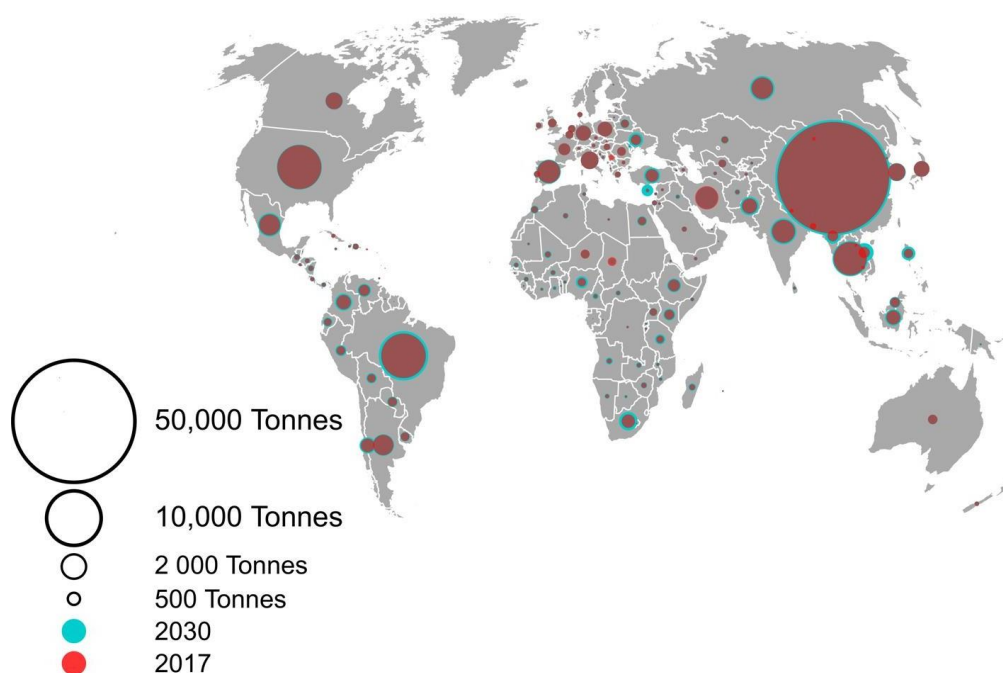
Figure 1. Estimated combined antimicrobial consumption in pigs, chicken and cattle per country in 2017 and 2030. The size of the circles corresponds to the amounts of antimicrobials used. Dark red circles correspond to the amounts used in 2017, and the outer blue ring corresponds to the projected increase in consumption in 2030. (Tiseo et al., 2020).

Antimicrobial resistance

There are no national monitoring data about antimicrobial resistance available from the countries in the region. Thus, one has to rely on scattered scientific reports of variable quality. Some of these studies are on pathogenic bacteria, whereas others are recording resistance in commensal *Escherichia coli (E. coli)* in similar way as in surveillance programmes elsewhere. One attempt to compile and display global surveillance data on AMR from scientific reports is the *reistancebank.org* -initiative (Cirscuolo et al. 20219).

Table 1 presents a sample of studies from various countries in the region on frequencies of resistance to common antibiotics in *E. coli* collected from pigs. Note that the studies in this table have not been in-depth analyzed nor harmonized with regard to design, farm-type, analytic method and statistic power, besides being peer-reviewed before publication in the respective journal. Thus, these data may not be extrapolated to the entire country and comparison between countries should be made with great caution.

Table 1. A sample of studies from East and Southeast Asia on frequencies of resistance to five antibiotics in *E. coli* collected from pigs.

Country	AMP	CIP	GN	CT	TET	Comment	Reference
China	80%	60%	55%	n.t.	90%	Meta-anlysis, including 16 studies published until February 2021	Li et al., 2021
Vietnam	85-100%	5-30%	18-45%	5-35%	n.t	Original study, samples collected 2013-14	Nguyen et al., 2016
Vietnam	80%	18%	28%	15%	80%	Original study, samples collected 2017-2019	Tuat et al., 2021
Vietnam and Thailand	90%	25%	38%	n.t.	95%	Review based on 12 studies, published 2000-20016	Nguyen et al., 2016
Thailand	70%	40%	11%	0%	75%	Original study, samples collected 2015	Ström et al., 2017
Thailand	n.t.	13%	6%	n.t.	58%	Original study, samples collected 2019	Lunha et al. 2020
Cambodia	70-85%	55-75%	25%	10-35%	75-90%	Original study, samples collected 2017	Ström et al. 2018

AMP, ampicillin; CIP, ciprofloxacin; GN; Gentamycin; CT:,Colistin; TET, Tetracyclin,; n.t. not tested

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A broad conclusion from these studies is that the frequencies of resistance in *E. coli* collected from pigs in the region to several commonly used antibiotics is very high. It could therefore be questioned whether it from a clinical perspective is meaningful to use them at all.

Antimicrobial Stewardship

Following overarching UN calls for action some 7-8 years ago, the countries in the region have developed National Action Plans on AMR, with emphasis on human health but also including parts on animal health (WHO, 2022). There is a variability in the ambitions between countries, but we do already see new regulations and other actions in place targeting the livestock sector. Those will, if properly implemented, likely have effects on both antimicrobial use and resistance in the pig sector.

Still, however, the controversial use of antimicrobials as growth promotors - as feed additives drive the development of antimicrobial resistance (Burrow et al., 2014) - is reported from 26% of OIE:s member countries, of which some of these are found in Asia (OIE 2021). However, it should be noted that there are newly launched regulations for the livestock sector about antimicrobial agents in several countries in the region. For instance, the ban of colistin for growth promotion in China 2016 (Shoenmaker, 2020) followed by a total ban of feed medication in 2019 (Chinese Ministry of Agriculture, 2019); in Thailand are antibiotics not allowed to be used as growth promoter since 2015 (Thai Ministry of Agriculture, 2015); Vietnam introduced its Animal Husbandry Law in 2018 banning the use of antimicrobials growth promoters in commercial feeds (Cuong et al., 2021).

Even so, it should be noted that in most of the countries in the region antimicrobials are readily available over the counter without prescription (Coyne et al. 2019; Zhang et al., 2022). In addition, the veterinary competence among the retailers is often low so the advice about use to the producer may be inappropriate or lacking (e.g., Phu et al., 2019; Heyman 2020).

Larger pig production operations in the region may either be directly company run or run as franchise by pig farmers. These operations do often have their own prophylactic or treatment guidelines (e.g., Hallenberg et al., 2020) that might be more restrictive or extensive regarding antimicrobial use than national guidelines. Also, the Association of Southeast Asian Nations has a Good Animal Husbandry Practice Guidelines, ASEAN GAHP, (Sirichokchatchawan et al., 2021) for improving animal health and reducing the need for antimicrobials.

As in all regions of the world, there might be an issue about the difference between regulations and the real world. Besides regulations and guidelines, there must be resources set aside to enforce these to reassure compliance. Another way to achieve compliance is to create positive economic incentives for the producers and other stakeholders that follow the regulations and guidelines.

Concluding remarks

Given the rapid economic development in the region, one foresees an increased demand for a more varied diet including animal sources foods. In response to this, the pig production is expected to increase further. However, if not measures against excessive and medically non-rational use of antimicrobials are applied, there is a high risk of further emergence of antimicrobial resistance that jeopardize animal health, welfare and productivity. Such measures include applying medically rational use of antibiotics and effective disease prevention without antimicrobial agents as shown to be cost-effective in other parts of the world; enforced regulatory frameworks about use and access to antimicrobials allowing economic incentives for producers and awareness campaigns among producers and consumers.

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Colonic innate immune defenses in swine dysentery caused by *Brachyspira hyodysenteriae*

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Introduction

Barrier function of porcine colon relies on organization of epithelial cells and mucus

The gastrointestinal tract lies at the interface between host and environment, absorbing nutrients and water into the body while sequestering foreign materials, toxins, pathogens, and commensal flora. As such, the pig's intestines form a crucial barrier against infection. This gut barrier is compromised during infectious colitis, allowing invading pathogens to colonize the intestines and trigger myriad host responses that attempt to clear the pathogen. Simplistically, intestinal host defenses may be divided into three broad categories: the epithelial and mucus barrier, innate immune responses, and the colonic microbiota (Figure 1).
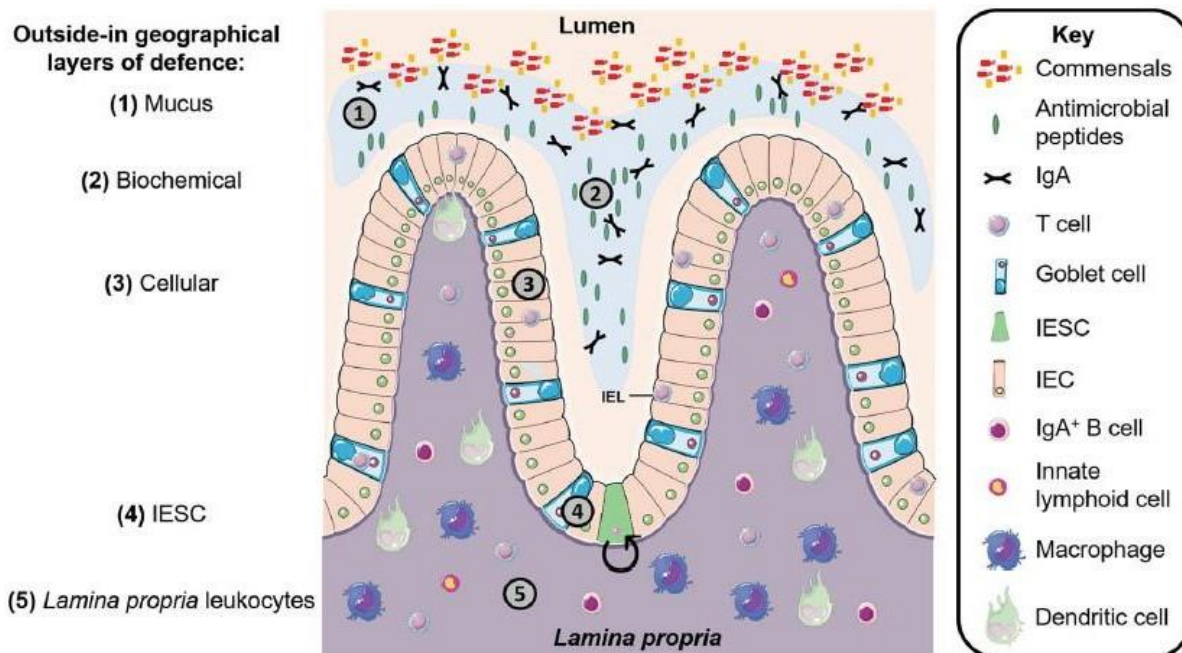
Figure 1. Intestinal host defenses. IESC: intestinal epithelial stem cell. IEC: intestinal epithelial cell. IgA: immunoglobulin A. Figure from previous publication (1).

The colon consists of folds of tissue (villi) separated by invaginations (crypts). Villi are composed of a monolayer of intestinal epithelial cells (IECs) and mucus-producing goblet cells, which derive from stem cells at the bottom of crypts and move toward the villus tip as they mature (2,3). IECs and goblet cells are connected by tight junctions that adhere each cell to its neighbours, forming a selectively permeable monolayer called the epithelium. The epithelium separates the intestinal lumen from the underlying tissue (lamina propria), where blood vessels and innate immune cells reside. Intestinal barrier function is compromised if the integrity of the epithelium is disrupted, allowing invasion of microorganisms into the lamina propria.

On the luminal side of the epithelium lies the colonic mucus, which forms a physical barrier designed to sequester luminal microorganisms away from host tissues. Mucus is formed mainly by glycoproteins called mucins that are composed of long protein chains covered in polysaccharides. Transmembrane mucins, particularly MUC1, 3, and 4

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in pigs, are expressed on the apical surface of IECs. The luminal portion of these mucins forms a dense glycocalyx that protects the cells, and the transmembrane portions may facilitate intracellular signaling in response to luminal stimuli. Covering the glycocalyx are mucus layers composed of secreted gel-forming mucins that are produced, stored, and secreted by goblet cells. Unlike the monomeric transmembrane mucins, secreted gel-forming mucins homo-oligomerize into large polymers that attract water, thus forming a hydrogel that coats the intestine. In the colon, mucus is separated in two layers. The inner layer is tightly adhered to the epithelium. This dense layer is reported as sterile because microorganisms cannot penetrate its small pores (4,5) and are killed by the constitutive presence of antimicrobial peptides (AMPs) (6). Host and bacterial enzymes degrade polysaccharide residues on mucins of the inner layer, which allows expansion of the mucin polymer to form a loosely-attached outer mucus layer (4). This outer layer is where commensal microorganisms reside. In pigs, colonic mucus is mainly composed of MUC2 but expression of MUC5AC may be induced during infection (7). The organization of colonic mucus and glycocalyx serves to protect epithelial cells from luminal microorganisms and toxins. When the mucus barrier is compromised, pathogens may come into direct contact with the epithelium and activate inflammatory host responses.

Inflammation initiates the recruitment of innate immune cells that clear infection

The presence of pathogens in the colon triggers inflammation (colitis), which is a signaling cascade that brings innate immune cells to the site of infection. IECs are important initiators of inflammatory responses because they come into direct contact with invading pathogens when the mucus barrier is compromised. IECs may sense the presence of microorganisms by a variety of mechanisms, including cell-surface pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) expressed by microorganisms (3). Transmembrane mucins may play a similar role in pathogen detection. PRR activation triggers intracellular signaling cascades within IECs that may result in secretion of antimicrobial peptides (AMPs) to kill the microbes in the lumen, and cytokines and chemokines to attract innate immune cells from the bloodstream into the lamina propria (3). Epithelial erosion may result from the pathogen itself or collateral damage from inflammatory responses (e.g., colitis caused by attaching/effacing pathogen *Citrobacter rodentium* in mice (8)). This may cause stem cells in the bottom of crypts to divide continuously (hyperplasia), which lengthens colonic crypts.

Neutrophils are considered the first innate immune cell to arrive at the site of infection with the primary goal of pathogen killing. Neutrophils destroy microorganisms using phagocytosis to engulf and digest pathogens, NETosis to trap them, and secretion of microbicidal compounds such as AMPs, myeloperoxidase (MPO) and reactive oxygen species (ROS) (9–12). Neutrophils also secrete chemokines and cytokines to further activate IECs and recruit more immune cells, thus perpetuating and amplifying inflammatory immune responses (13). Monocytes are recruited from the bloodstream then differentiate into macrophages inside colonic tissue, where they destroy pathogens using similar mechanisms to neutrophils (e.g., phagocytosis, ROS, cytokine production) (14). However, macrophages also participate in wound healing and tissue repair by phagocytosing dead cells and debris, and can initiate adaptive immune responses by antigen presentation to T and B cells in the lymph (14,15).

Colonic microbiota modulates host defenses by interacting with mucus, innate immune cells, and pathogens

The outer mucus layer is colonized by luminal commensal microorganisms that make up the colonic microbiome. These microorganisms use the mucus layer as a habitat, expressing lectins to attach to glycans on mucin molecules and finding food by consuming both glycans and ingested nutrients in the lumen. The repertoire of mucin glycosylation patterns possessed by the host may influence the composition of the microbiota and vice versa, which influences pathogenesis of infectious disease. Commensals strengthen the immune system by secreting metabolites such as butyrate, which promotes epithelial barrier integrity (16,17) and stimulates secretion of AMPs (18). Commensals may further strengthen host defenses by consuming nutrients and occupying space, thereby competing against pathogens. This is demonstrated by murine models that require antibiotic pre-treatment before infection with *Clostridium difficile* (17,18). Alternatively, commensals may contribute to pathogenesis by creating favorable environments for pathogen colonization (e.g., by secretion of metabolites as pathogen food sources or altering mucin glycosylation). Commensals normally sequestered in colonic lumen may even become opportunistic pathogens themselves when disruption of the mucus/epithelial barrier brings them into direct contact with host cells.

Swine dysentery as a model for colonic innate defenses against bacterial infection

Swine dysentery (SD) is an infection of grower-finisher pigs with the enteric anaerobic spirochete *Brachyspira hyodysenteriae*. The hallmark clinical sign of SD is mucohemorrhagic (bloody) diarrhea, often leading to poor feed conversion, stunted growth and up to 30% mortality in naturally infected pigs (19). Remarkably, evidence from experimental infection studies suggest that colonization with *B. hyodysenteriae* alone is not always sufficient to produce clinical signs of disease. Thus, interplay between pathogen, host, and environmental factors may be required to produce clinical SD, offering a unique opportunity to study colonic innate defenses in infectious colitis.

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Although muco-hemorrhagic diarrhea is considered the hallmark clinical presentation of SD, literature reports that incidence of bloody diarrhea in experimentally infected pigs is variable and rarely reaches 100% (20). Most experimental inoculation studies report hemorrhagic diarrhea incidence ranging from 33% to 92% (21–27), while others fail to induce hemorrhagic diarrhea at all, producing only milder diarrhea without blood (20,24,28). Despite rigorously controlled experimental conditions, the incubation period between exposure and onset of clinical signs is also variable (1- 4 weeks) and some infected pigs may remain asymptomatic while still transmitting the pathogen by fecal shedding. This demonstrates that clinical presentation of SD is variable among pigs: some pigs may be particularly susceptible to *B. hyodysenteriae* infection whereas others are resilient. Research to date has often compared challenged and unchallenged pigs regardless of diarrhea severity and individual host responses. Thus, it remains unknown why some challenged pigs become diseased while others are non-diseased, and whether gut innate defenses influence clinical outcomes.

To understand host/intestinal factors that impact pathogenesis and their relation to disease severity, we conducted experimental infection of pigs with *B. hyodysenteriae*. Like literature, we showed a proportion of challenged pigs were diseased (developed bloody diarrhea, clinically affected with SD) but others were non-diseased (did not develop bloody diarrhea). Of the non-diseased pigs, some were colonized with *B. hyodysenteriae* while others were not, but all diseased pigs were colonized (Figure 2a). Comparing *non-diseased colonized* and *diseased colonized* pigs allowed us to identify host factors involved in pathogenesis of clinical SD. Diseased pigs displayed microscopic colonic lesions that were absent in non-diseased pigs regardless of *B. hyodysenteriae* colonization status (Figure 2b). They showed longer crypt depth (hyperplasia), erosion of the epithelium, hemorrhage in the lamina propria, and infiltration of immune/inflammatory cells in the colonic lumen and near the epithelial surface. These results indicate that pigs may be infected with *B. hyodysenteriae* yet remain asymptomatic and without histological colitis.
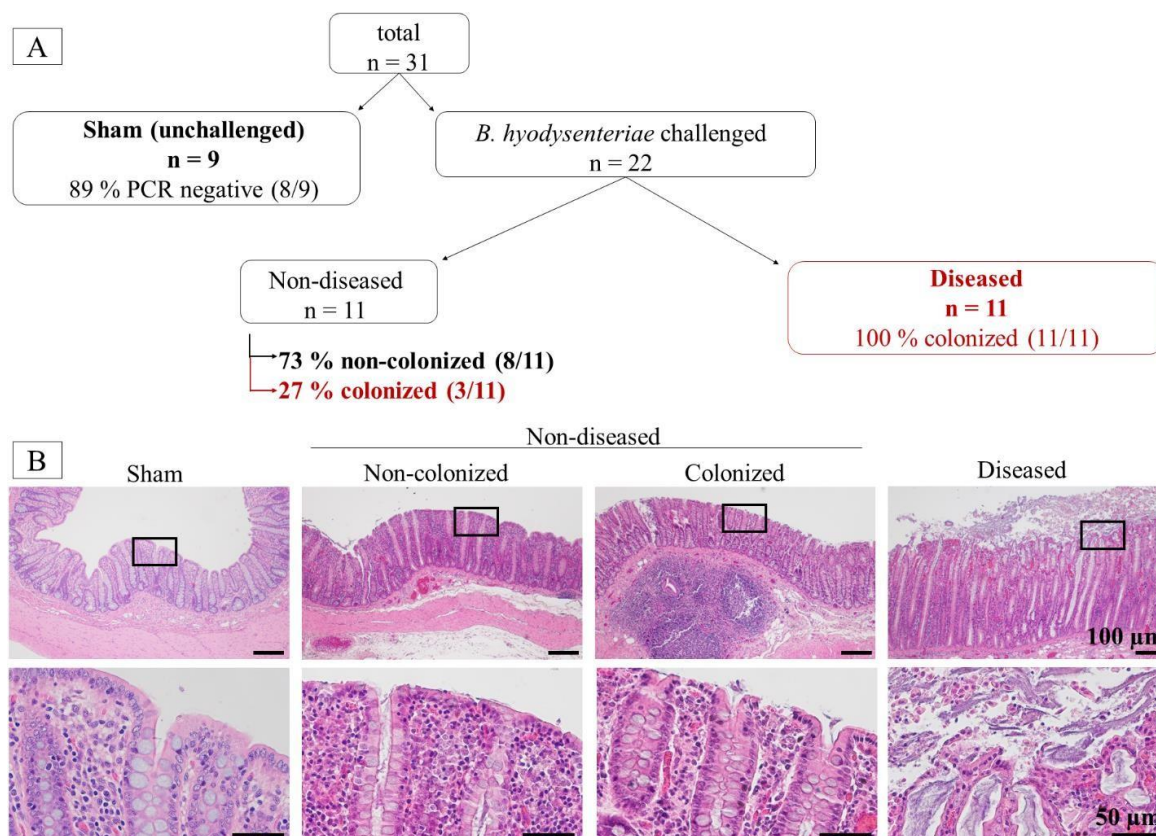
Figure 2. Pigs experimentally infected with *Brachyspira hyodysenteriae* show variation in severity of diarrhea and colitis. A) Breakdown of animal groups. Pigs that develop bloody diarrhea are "diseased". Pigs that are challenged with *B. hyodysenteriae* yet do not develop bloody diarrhea are "non-diseased". Detection of *B. hyodysenteriae* from colonic digesta by qPCR further divides non-diseased pigs into colonized and non-colonized groups. **B)** Representative images of H&E-stained colon sections.

Intestinal mucus alterations may promote *B. hyodysenteriae* colonization

SD causes mucus hypersecretion in the gut (21,29–31), unlike other colonic bacterial infections that degrade mucus. Mucus hypersecretion may function as a host defense mechanism to flush out pathogens. However, this strategy may be useless against SD because *B. hyodysenteriae* is well-adapted to thrive in a mucus-rich environment (32,33),

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illustrating the evolutionary arms race between pig and pathogen. Whether mucus hypersecretion in SD is driven by host or pathogen has been debated. In our study, diseased pigs showed alterations in the colonic mucus environment. Alcian blue staining showed fewer filled goblet cells, crypts filled with mucus, and excess mucus secretion into the lumen in colons of diseased pigs (Figure 3a). This corresponded with a trend in gene upregulation of MUC2 and MUC5AC (data not shown), and mobilization and increased presence of sialylated mucins into crypt and colonic lumens (Figure 3b). The finding that non-diseased pigs colonized with *B. hyodysenteriae* do not display mucus alterations infers such mucus changes are mostly host-driven rather than promoted by the pathogen itself.

B. hyodysenteriae consumes sialic acid monosaccharides as a food source, and glycan patterns containing sialic acid were more abundant in pigs with SD than unchallenged pigs (34). Furthermore, there is greater variation in the repertoire of glycan patterns between individual unchallenged pigs than between pigs with SD (30). Taken together, these data indicate that host-driven changes in mucus composition and mucin glycosylation with greater sialic acid content may drive susceptibility to *B. hyodysenteriae* infection.
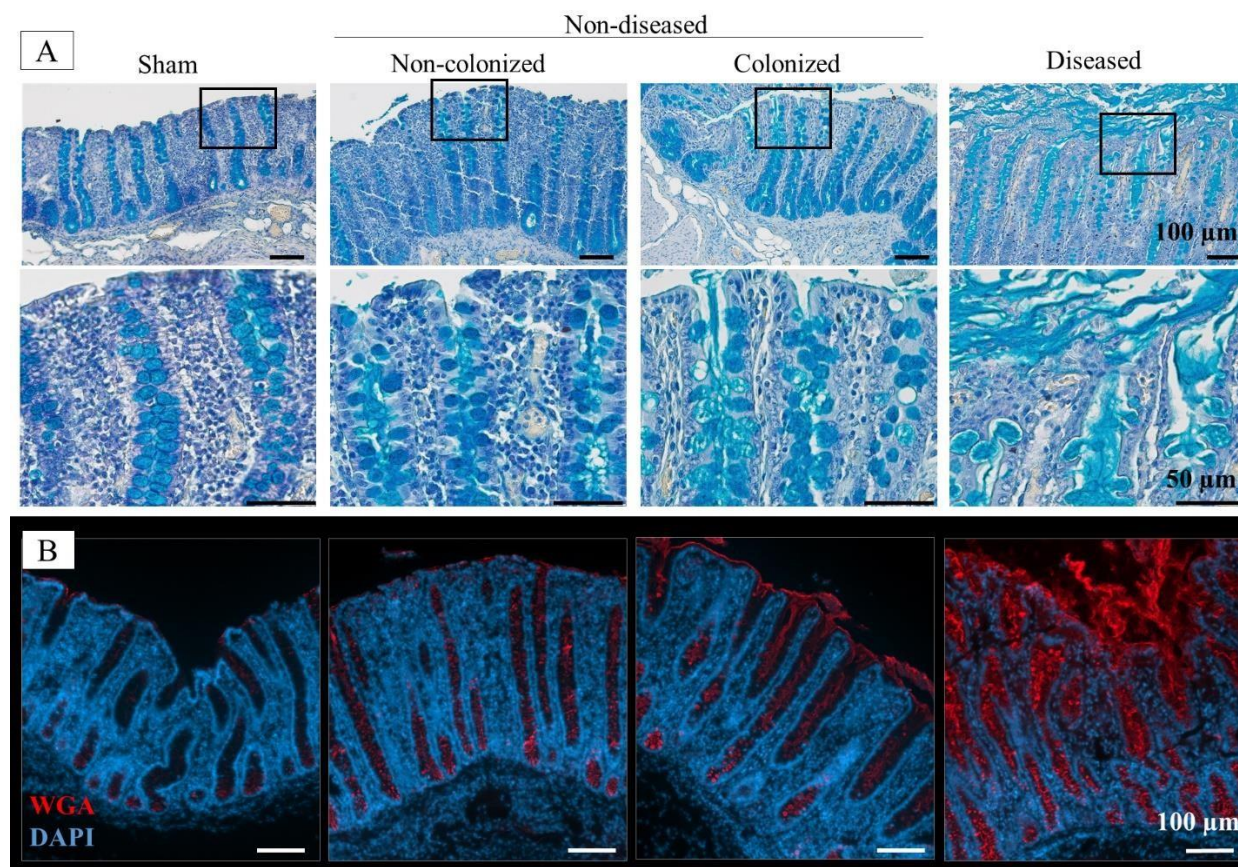
Figure 3. Diseased pigs display colonic mucus alterations not observed in non-diseased pigs. A) Representative images of Alcian blue-stained colons. **B)** Representative immunofluorescent images of colons stained with WGA lectin to detect N-acetylglucosamine and sialic acid (red), counterstained with DAPI (blue).

Neutrophils and macrophages did not clear *B. hyodysenteriae* despite AMP expression

Early recruited leukocytes are a key defense against infection, and neutrophilic infiltrates are commonly reported in colons of clinically affected pigs with SD (21,29). We showed neutrophils in diseased pigs localized in the lumen and near the epithelial surface (Figure 4). This is where neutrophils would encounter *B. hyodysenteriae,* since the pathogen localizes within colonic mucus by binding to sialic acid residues (29,34), attaching to epithelial cells (35), or internalizing within goblet cells (30). The fact that neutrophils are strongly detected in diseased but not in non- diseased pigs regardless of *B. hyodysenteriae* colonization status suggests that neutrophils are associated with clinical signs of SD. Gene expression of CXCL8, the classical chemokine for neutrophil recruitment (36), was similar among all groups (data not shown) indicating that neutrophil recruitment in SD is by other CXCL8-independent mechanisms or by secretion of pre-formed cytokine without transcriptional regulation.

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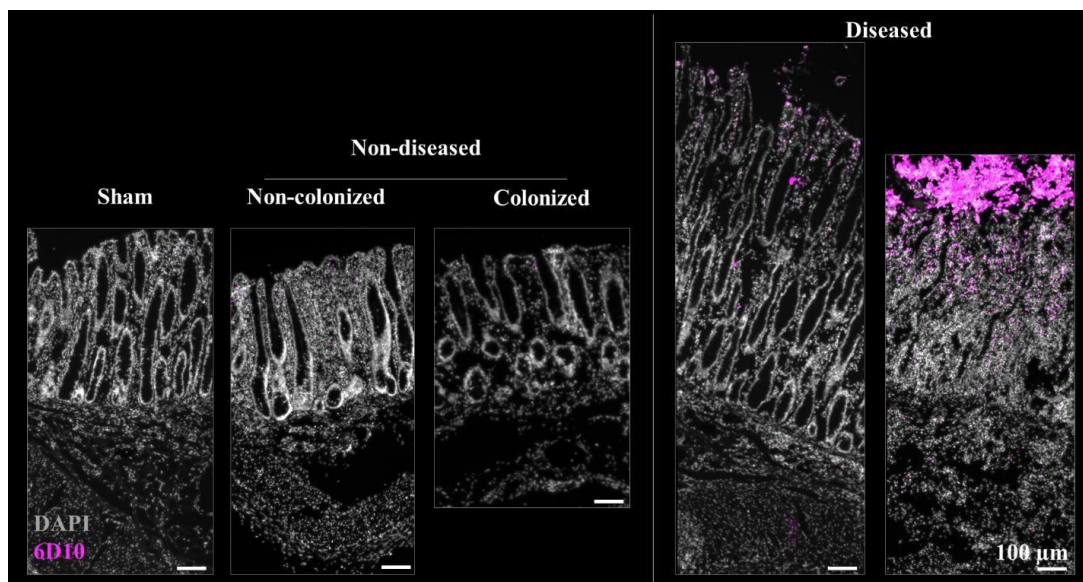
Figure 4. Neutrophils localize near colonic epithelium and lumen in diseased pigs. Representative immunofluorescent images of colon cryosections stained for the 6D10[+] neutrophil marker (magenta) and counterstained with nuclear DAPI (gray).

We analyzed whether macrophages may be providing bacterial clearance defenses in the colon. We observed macrophages located at the tops and bottoms of colonic crypts in both sham and diseased pigs **(Fig 5)** suggesting macrophages do not impact clinical SD. However, non-diseased non-colonized pigs tended to have macrophages localized throughout the colonic lamina propria. Furthermore, non-diseased colonized pigs varied greatly, from having macrophages distributed throughout the lamina propria (like non-diseased negative group) to very few macrophages detected whatsoever. Thus, the presence of macrophages in the colon may be associated with the reparative/wound healing phase of infection (as in the non-diseased non-colonized group), or indicative of early infection stages in pigs without mucohemorrhagic diarrhea (non-diseased colonized group).

It is important to note that both neutrophil and macrophage populations in this study were examined only at a single timepoint at the peak of clinical signs of SD (upon bloody diarrhea onset). Thus, efficacy of bacterial clearance by neutrophils and macrophages could not be determined since this would require detecting cell populations at different time points during infection to associate their presence with bacterial shedding. However, previous studies showed that circulating blood neutrophils and monocytes increase during the onset of clinical signs of SD, and remain elevated until recovery after clinical signs have ceased (22,23). The dysentery and recovery periods were not necessarily associated with pathogen clearance since some pigs continued to shed *B. hyodysenteriae* in feces during and after recovery (22). Thus, innate immune defenses by neutrophils and macrophages may drive clinical presentation (diarrhea) instead of pathogen clearance.
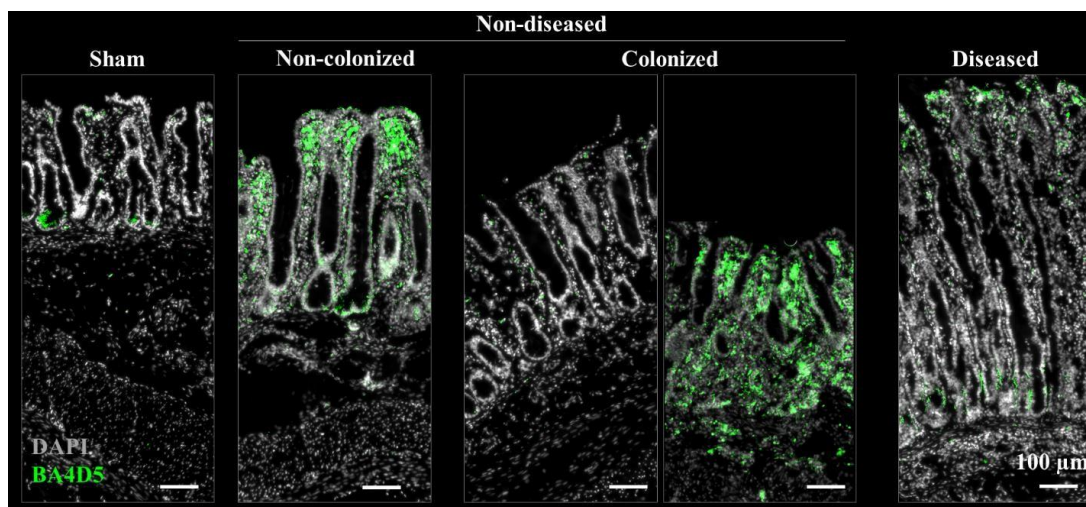
Figure 5. Colonic macrophages are distributed differently depending on clinical signs of disease. Representative immunofluorescent images of colon cryosections stained for the BA4D5[+] macrophage marker (green) and counterstained with nuclear DAPI (gray).

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We assessed antimicrobial effectors to further elucidate the ability of neutrophils to clear *B. hyodysenteriae* infection. Colonic myeloperoxidase (MPO), abundantly produced by neutrophils and macrophages, was quantified as a measure of degranulation (37). MPO activity did not differ between diseased and non-diseased groups. Colonic gene expression of cathelicidins, small antimicrobial peptides with immunomodulatory pro-inflammatory functions produced by neutrophils, were also assessed. Cathelicidins PMAP-37 was detected in 90% of diseased pigs compared to up to 67% in other groups, while PR-39 and protegrins 1-5 were not clearly associated with clinical signs of SD. Thus, PMAP-37 is activated as part of innate immune cell defenses (but not MPO or other cathelicidins) and is unable to clear *B. hyodysenteriae*.

Microbiome 16S sequencing indicates B. hyodysenteriae requires co-infection and is susceptible to competition from Lactobacillus

We explored the colonic microbiome associated with the mucosa and with the luminal contents (digesta) separately. In all parameters explored, the colonic mucosa showed more differences than colonic digesta, perhaps indicating invasion of commensals in the normally sterile inner mucus layer. In general, sham (normal microflora) and non- diseased groups showed similar microbiome profiles to each other but differed from diseased pigs. Diseased pigs showed dysbiosis in colonic mucosal microbiota, indicated by reduced alpha diversity. In colonic mucosa and digesta, beta diversity based on Bray-Curtis distance (a measure of diversity between samples) showed diseased pigs have an unique bacterial profile at genus level (Figure 6). A direct comparison of diseased and non-diseased pigs identified *[Acetivibrio] ethanolgignens* as characteristic in diseased pigs. *A. ethanolgignens* was originally discovered when isolated from pigs with SD (38,39) but has not been described since. It is possible that co-infection with this pathogen is required for clinical SD. *Lactobacillus* showed to be characteristic of non-diseased pigs, confirming similar findings in a previous study (40). This indicates promise for probiotic prevention strategies against SD. Bacterial taxa were matched to predicted functional categories in the KEGG database (41) which found differences in metabolic pathways among pig groups. Thus, changes in colonic microbiota composition may provide a mechanism to explain previous reports that diet affects SD susceptibility (42–49).
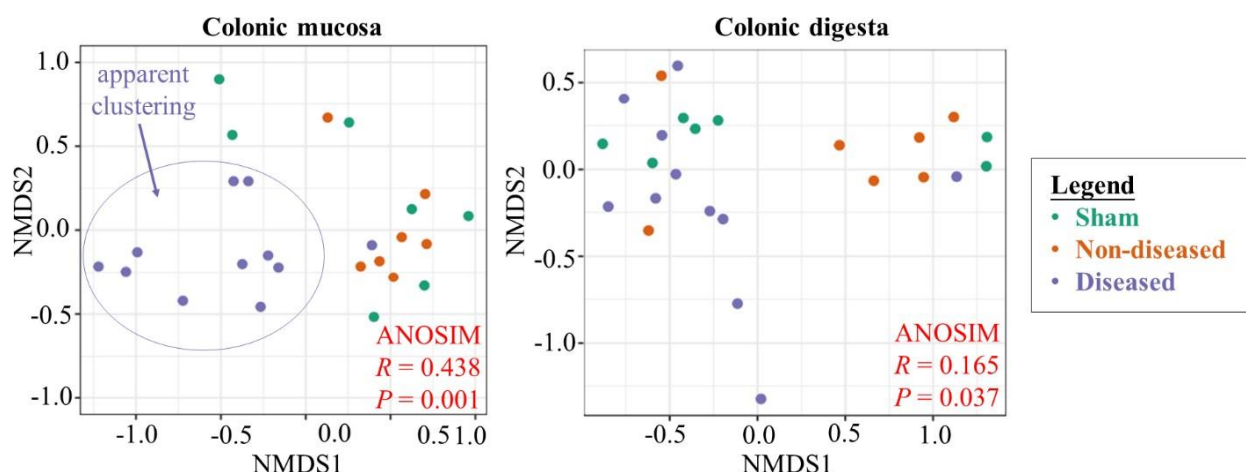
Figure 6. Beta diversity based on Bray-Curtis distance of bacterial profiles at genus level identified by 16S sequencing. Each data point represents one animal. Clustering of datapoints suggests similarity while dispersion suggests differences between animals. $\alpha = 0.05$.

Cathelicidins are potential therapeutic modulators of colonic defenses in SD

Cathelicidins are a group of host defense peptides in mammalian innate immunity, secreted by leukocytes and differentiated epithelial cells at the surface of colonic crypts (50–53). All mammals express cathelicidins, but mammalian species like humans and mice contain a single gene for an alpha-helical peptide: LL-37 and CRAMP, respectively (54). Pigs are an exception and express nearly a dozen cathelicidins with a variety of structural conformations, including proline-arginine-rich 39-amino-acid peptide (PR-39), protegrins (PGs) -1 to -5, and porcine myeloid antimicrobial peptides (PMAPs)-23, -36 and -37 (50,51,54). Although not tested against *B. hyodysenteriae,* these peptides possess microbicidal activity against a variety of Gram-negative pathogens (54). The mechanism of microbicidal activity depends on the peptide and may be lytic (PGs, PMAPs) or non-lytic by internalizing into cells and blocking DNA synthesis (PR-39) (54). However, evolutionary evidence suggests that the function of cathelicidins is immunomodulation and not merely microbicidal activity. Peptide regions responsible for antimicrobial activity are not evolutionarily conserved among mammalian species (55). Conversely, the portions of

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cathelicidin precursors that are indispensable for immunomodulation are highly conserved and co-evolved with the pathogen recognition formyl peptide receptor (FPR2) (55). Thus, mammalian cathelicidins may be integral in innate defense against enteric infection and porcine cathelicidins regulate many aspects of host responses in colitis. The colonic mucus layer is regulated by cathelicidins, demonstrated when PG-1 attenuated murine DSS colitis by increasing MUC2 gene expression, preventing goblet cell loss and restoring the colonic mucus barrier (56). Furthermore, the most well- studied porcine cathelicidin, PR-39, promotes pro-inflammatory neutrophil and macrophage functions. PR-39 binds neutrophil DNA to protect NETs from enzymatic degradation (57) and is directly chemo attractive for neutrophils (58,59) but not mononuclear cells (59). PR-39 also promotes IL-8 and TNF-α secretion by immortalized porcine alveolar (3D4/31) macrophages (60). Thus, cathelicidins could mediate colitis by modulating innate immune cell and goblet cell function.

Cathelicidins have not been explored in SD. However, their immunomodulatory activities warrant their investigation as a possible therapeutic, which may reduce or replace antibiotic use against this increasingly resistant disease. It is possible that *B. hyodysenteriae* has become resistant to host defenses mediated by endogenous porcine cathelicidins, so we focussed on the murine cathelicidin CRAMP as a primary candidate for a novel therapeutic. CRAMP has never been administered to pigs, so we explored its safety in naïve pigs unchallenged to *B. hyodysenteriae*. A pilot study showed CRAMP intraperitoneal injection was well tolerated for over 2 weeks, with no signs of histological colitis and minimal mucus barrier alterations. Future exploration of doses, practical routes of administration, and efficacy in a *B. hyodysenteriae* infection model may elucidate abilities of CRAMP to strengthen mucus, innate immune and microbiome defenses against SD.

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Stimulating a protective adaptive immune response
to *Lawsonia intracellularis* in pigs

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Lawsonia intracellularis-based diseases in pigs

Proliferative enteropathy (PE) is an enteric infectious disease caused by the obligate intracellular Gram-negative bacteria *Lawsonia intracellularis* (McOrist et al. 1993; Biester and Schwarte 1931). This pathogen is endemic throughout the world and it is a major cause of weight loss, mortality in pigs and significant economic losses for the swine industry (McOrist 2005). PE in pigs has two distinct clinical and pathologic forms, porcine intestinal adenomatosis (PIA) and proliferative hemorrhagic enteropathy (PHE). Porcine intestinal adenomatosis (PIA) affects young, post-weaned pigs, usually between 6 and 20 weeks of age and the major symptoms include diarrhea, anorexia and weight loss (Vannucci and Gebhart 2014). This disease is often mild, and it is subclinical in form with major characteristics including thickening of the wall of intestine due to proliferating immature crypt epithelial cells (McOrist et al. 1996). Evidence of infection is usually only observed at slaughter when pathologic lesions can be observed in the alimentary tract, especially in the terminal ileum. Despite these lesions, the pig immune system does not respond to infection with infiltration by inflammatory cells at this stage of the disease, which may indicate that the bacteria have an immunosuppressive effect on the host's immune system (Lawson and Gebhart 2000). Although clinical signs are mild, infected animals shed the bacteria in the feces and are a source of disease transmission. Despite the overt lack of clinical signs of disease, PIA impedes weight gain, thus negatively affects barn profitability, making it an important swine disease. PHE is the acute form of the disease that mainly affects young, adult pigs, 4 to 12 months of age such as finishing pigs, gilts, and boars (Kroll et al. 2005). The main symptoms of PHE are profuse hemorrhagic diarrhea leading to the sudden death of the animals. The pathological findings include thickened and rugose mucosa of the terminal ileum replete with blood in the intestinal lumen. Histological analysis indicates that the ileal crypts have undergone extensive proliferation and there may be evidence of bacteria in the apical cytoplasm of the epithelial cells, in macrophages in the *lamina propria* and submucosa, within epithelial capillaries, and within the lymphatics (Guedes et al. 2017; Rowland and Lawson 1974). An obvious inflammatory response is present in the PHE form of the disease, and this inflammatory response may be the reason for more severe symptoms and high mortality rate relative to the PIA form of the disease.

The adaptive immune response

The mammalian intestinal adaptive immune system has evolved to provide antigen-specific memory responses to intestinal pathogens, while at the same time maintaining tolerance to environmental, food or commensal antigens. Innate immune cells in the intestinal mucosa provide the necessary signals to respond to antigens which is communicated to and impacts the cells of the adaptive immune system. Adaptive immunity in the gut consists mainly of humoral immunity mediated by secreted IgA antibodies, induction of protective cell-mediated adaptive immunity mediated by CD4+ and CD8+ T cells, and induction of Treg cells that promote gut homeostasis and tolerance to food antigens (Gormley et al. 1998; Kiyono and Fukuyama 2004). The Peyer Patches and lymphoglandular complexes of the gut-associated lymphoid tissues (GALT) are the primary inductive sites in the gut, but the functions of the isolated lymphoid follicles and cryptopatches are unclear (Cesta 2006). The maturation of GALT depends on the presence of commensal bacteria and their products (Goto and Ivanov 2013). The relationship between microbiota and immune cells in GALT is bi-directional with both host and commensal bacteria cells contributing to immune homeostasis.

GALT is rich in effector T cells and it is the major site of secretion of antigen-specific IgA (Gormley et al. 1998; Kiyono and Fukuyama 2004). IgA is the main mediator of specific humoral immune protection, it is non-inflammatory and an important regulator of the commensal population (Pabst 2012). IgA antibodies are produced by plasma cells that stem from activated B lymphocytes which have undergone class-switch recombination in GALT (Pabst 2012). IgA antibodies are bound by polymeric immunoglobulin receptors (pIgRs) on the basolateral membrane of intestinal epithelial cells (IECs) and are then actively transcytosed to the apical surface and released into the lumen as secretory IgA (sIgA) (Pabst 2012; Johansen and Kaetzel 2011). Once in the lumen, sIgA can bind to pathogens or toxins and prevent their interaction with intestinal epithelial cells (Mantis and Forbes 2010). Alternatively, IgA can bind

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pathogens in the *lamina propria* then bind to pIgR on the basolateral surface of the IECs resulting in transport and the release of the pathogen into the lumen. Intestinal IgA antibodies comprise around approximately 70% of all antibodies produced in mammals, making it the most abundant antibody class (Macpherson et al. 2008). Oral vaccination that target activation of antigen-specific long-lived IgA secreting plasma cells may be a feasible strategy to induce long-lasting protection against enteric pathogens while at the same time maintaining necessary immune balance in the intestinal mucosa.

The cell-mediated immunity in the intestine

Adaptive cell-mediated immunity is mediated by T cells present in GALT including in close proximity to the intestinal epithelium. T-cells are differentiated by the expressed surface receptors CD4^{+} and CD8^{+} cells. CD4+ T cells can be distinguished into distinct subsets by their distinct effector function and cytokine secretion in response to the antigen and co-stimulatory molecules presented on APCs or cytokines secreted in the intestinal epithelial environment. They play multiple roles in immune protection against intestinal pathogens and they play an important role in maintaining gut homeostasis in an environment rich in food and commensal antigens.

CD4 helper T cells (T$_H$) recognize antigens presented in major histocompatibility complexes class II (MHCII) after processing by APCs (Konjar et al. 2017). CD4+ T$_H$ cells further differentiate into effector cells with specific functions in Th1, Th2, and Th17 immunity with cellular plasticity in intestinal tissue (Brucklacher-Waldert et al. 2014). Th1-type T-cells produce IFNγ cytokine, Th2-type T cells produce IL-4, IL-5, and IL-13 (Mosmann and Coffman 1989) and Th-17-type T cell cells produce IL-17A and IL-17F and IL-22 which induce neutrophil production and promote maintenance of epithelial barrier integrity (Weaver and Murphy 2007; Veldhoen et al. 2006). CD4+ regulatory Foxp3 expressing T cells (Tregs) are a subset of T cells that play a major factor in immune regulation and tolerance and a high proportion of these subsets reside in intestinal *lamina propria* (Agace and McCoy 2017). CD8 expressing cytotoxic T cells recognize antigens presented in MHCI on host cells, deriving from viral or intracellular bacterial pathogens that infected host cells. When CD8^{+} T cells engage with MHCI presenting intracellular antigen, they are activated and they differentiate into effector cells which results in the production of IFNγ or they have cytotoxic activity directed towards infected cells. IFNγ is a strong activator of innate immune cells such as macrophages and an inducer of class-switch recombination of B cells to immunoglobulin IgG isotypes that opsonize microbes for enhanced uptake by phagocytes (Weaver et al. 2013).

The gut epithelium is home to an abundant population of CD8^{+} T cells located in between epithelial cells or dispersed in underlying tissue (Kato et al. 2014). The CD8^{+} T-cells are distinguished by expression of αβ or γδ TCRs, which determines their specific localization and role in the intestinal immune system (Gerner, Kaser, and Saalmuller 2009). The γδ T-cells are intraepithelial lymphocytes (IELs) while αβ T-cells are located in the *lamina propria*. Subsets of γδ T-cells are present in low numbers in the circulation but they are abundant in epithelial tissues and constitute between 10–100% of T-cells in the epidermis of the skin and the epithelial tissues in gastrointestinal tract (Nielsen, Witherden, and Havran 2017). They play an important role in maintaining epithelial barrier, regeneration of epithelium, homeostasis and providing a balance between commensal tolerance and pathogen clearance (Nielsen, Witherden, and Havran 2017).

L. intracellularis vaccine development

Currently, there are two registered vaccines against *L. intracellularis* available for prevention of PE in pig farms. The first registered vaccine against *L. intracellularis* (Enterisol, Boehringer Ingelheim) was an avirulent live vaccine developed by multiple consecutive passaging of bacterial isolate B3903 in McCoy cells (Kroll, Roof, and McOrist 2004). The attenuation of virulence is achieved between 20 and 40 passages *in vitro* which is represented by attenuation and down-regulation of important genes responsible for virulence and cell metabolism in bacteria (Vannucci et al. 2013). The vaccine is administered orally to animals and each dose is comprised of approximately 10^5 bacteria (Kroll, Roof, and McOrist 2004). After challenge with virulent bacteria, vaccinated pigs showed partial protection of pigs with a significant reduction in intestinal lesions, the absence of clinical symptoms, and higher average daily gains compared to the unvaccinated control group (Kroll, Roof, and McOrist 2004). Although fecal shedding was reduced in vaccinated group (47% and 40% reduction on day 35 and 42, respectively), vaccinated animals still shed bacteria in great numbers and therefore may be a source of transmission of *L. intracellularis* in naïve pigs (Kroll, Roof, and McOrist 2004). A study from Nogueira et al 2013 compared prescribed 1x oral dose ($10^{4.9}$ TCID$_{50}$) to 10x oral ($10^{5.9}$ TCID$_{50}$) and 1x intramuscular (i.m.) dose and found that pigs that received 10x higher oral dose showed increased serum and mucosal concentrations of IgM and IgG antibodies, increased TNF-α and TGF-β1 in the intestinal mucosa that trended towards higher levels of serum IFNγ and IL-6 on day 17 (Nogueira et al. 2013).

Levels of serum IgG titers were increased between day 9 and day 17 for all vaccinated groups and those levels were similar for oral and 1x i.m. vaccinated animals. In contrast, serum and mucosal levels of IgA titers were below detection limits in all vaccinated groups (Nogueira et al. 2013).

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Vaccinated and control animals were challenged orally with pathogenic bacteria (25 ml of a suspension containing around 10^9 bacteria) and after challenge, the control and all groups of vaccinated animals had an increase of serum cytokines levels IFNγ, IL-6, IL-10 and TNF-α from day 0 to day 21 post-challenge and significant increases of serum *L. intracellularis* specific IgG and IgA (Nogueira et al. 2013). Although all vaccinated pigs in this trial had significantly reduced fecal shedding of *L. intracellularis* after virulent challenge compared to non-vaccinated control group, animals vaccinated with 10x dose shed significantly fewer bacteria in their feces and had less percentage of the affected area in the ileum than other 2 vaccinated groups (Nogueira et al. 2013). The observed immune protection of oral 10x dose might be attributed to stronger priming of both cell-mediated and humoral immunity in intestinal mucosa than a 1x oral dose or 1x i.m. dose. Intramuscular administration of 1x dose of live avirulent vaccine was also able to provide a protection from virulent challenge by reducing fecal shedding and intestinal pathology suggesting that serum antibodies might play role in preventing entry of bacteria into the cells or by antibody-dependent cell- mediated cytotoxicity (ADCC) (Nogueira et al. 2013).

A second commercially available vaccine against *L. intracellularis* contains inactivated whole cell bacteria in an oil-in-water emulsion and adjuvant based on mineral oil and alpha-tocopherol (Vitamin E) (Roerink et al. 2018). (Porcilis Ileitis, Merck Animal Health, Madison, NJ, USA). Protection properties have been assessed under field conditions when the vaccine was administered intramuscularly to 22-25 days old pigs, just before weaning (Roerink et al. 2018). The vaccine induced significant serum antibody titres and upon subsequent challenge, these animals had 15-fold reduced bacterial fecal shedding and significantly reduced bacterial burden and microscopic lesions in ileum compared to control animals (Roerink et al. 2018). Vaccinated animals had preserved gut integrity and preserved goblet cells suggesting that the vaccinated animals had reduced incidence and severity of clinical symptoms of PE, and reduced colonization and duration of fecal bacterial shedding (Roerink et al. 2018). In a field study trial, 75 piglets were allotted to three groups vaccinated at 4 or 5 weeks of age with either Porcilis® Lawsonia in adjuvant or mixed with Porcilis® PCV M Hyo vaccine (group 1), with Enterisol (group 2), or unvaccinated (group 3) (Jacobs et al. 2019). They were challenged 3,4 or 17 weeks post-immunization. Pigs vaccinated with Porcilis® Lawsonia demonstrated by lower clinical scores, improved weight gain, reduction of Lawsonia intracellularis shedding and reduction of macroscopic as well as microscopic ileum lesion scores when compared to the controls relative to the live vaccine (Jacobs et al. 2019). Studies need to be undertaken to determine whether the immune correlates of protection include induction of humoral immunity response.

The above commercial vaccines represent a live, attenuated and a killed vaccine, respectively. However, subunit vaccines are an attractive option for vaccine development because they are very safe (they cannot revert to virulence), the type of immune response induced can be modified by co-formulation with adjuvants, and because only 1 or at most a few antigens are usually included, one can look at serum antibodies to discern whether the animals have been vaccinated (i.e. antibodies against only select antigens are present) or whether they have been infected (antibodies against numerous *L. intracellularis* antigens will be present).

Subunit B cell antigen selection and analysis

To develop a subunit vaccine, we need to identify immunogenic proteins to include. While the *L. intracellularis* antigenic properties have still not been fully elucidated, several proteomic studies have been performed. On an SDS-PAGE gel, *L. intracellularis* has 25 to 27 visible protein bands, from which 22 are conserved in six different isolates (McOrist et al. 1995). The protein masses of the major bands are 53 kDa, 42 kDa, 37 kda and 30 kDa (McOrist et al. 1995). The serum polyclonal 1999PAb antibody targets five outer membrane proteins (OMPs) with masses of 77, 69, 54, 42 and 36 kDa while the monoclonal 2001MAb targets OMP of 77 kDa, and monoclonal IG4 targets a protein of 18 kDa (Guedes and Gebhart 2003). Other select proteins that constitute the outer membrane have been detected and characterized such as LsaA (27 kDa), which is involved in cell attachment and invasion (Guedes and Gebhart 2003) and LatA autotransporter protein (72 kDa) (Watson et al. 2011). LsaA is predicted to have role in cell attachment and invasion and is expressed during *in-vitro* infection of IEC-18 cells and also in infected enterocytes from ileum tissue (Guedes and Gebhart 2003). This antigen is recognized by the monoclonal antibody VPM53 using IHC detection in both IEC-18 cells and tissue samples (Guedes and Gebhart 2003). Based on its similarity to proteins of other Gram-negative bacteria, LsaA is believed to play a role in pathogenicity. Further studies are needed to characterize its function and cellular networks. The outer membrane protein LatA, (LI0649) belonging to the family of autotransporter proteins was detected using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (Watson et al. 2011). The predicted LatA molecular mass was 91.9 kDa but a 72 kDa form of LatA was observed on SDS-PAGE gels which implies that protein cleavage occurred (Watson et al. 2011). This gene was cloned and the purified protein was recognized by the immune serum from pigs infected with *L. intracellularis* indicating that is antigenic (Watson et al. 2011). This protein is highly specific to *L. intracellularis* and it can be exploited for development of diagnostic tests for PE.

The same researchers applied shotgun proteomic analysis on two *L. intracellularis* isolates and identified 19 proteins, including LI0841 and LI0902 that were predicted as outer membrane-associated proteins (Watson et al. 2014). The

rLI0841 protein was identified as a putative invasin with sequence similarity to proteins that promote invasion. The rLI0902 protein was predicted to be involved in protein-protein interactions. Both recombinant proteins were recognized by serum antibodies from infected pigs (Watson et al. 2014). Recognition of these two recombinant proteins by pig immune sera indicate that they are expressed and available to host immune recognition thus making them potentially good diagnostic tools or antigens for a subunit vaccine. *L. intracellularis*, like all Gram-negative bacteria, have lipopolysaccharide (LPS) in the outer membrane which has been used as an antigen target for ELISA antibody detection in pig serum (27). LPS based ELISA are not ideal diagnostic tools due to the high cost of LPS antigen extraction and differences in background LPS reactivity among animals.

Analysis of 20 genes highly expressed by *L. intracellularis* when it resides in the enterocyte cytoplasm led to identification of 7 unknown genes whose corresponding proteins are part of the outer membrane surface (Won and Lee 2018). These hypothetical OMPs could play roles in attachment and invasion of host cells and thus might be recognized by the immune system of the host. Further investigation into their immunogenicity is needed before it can be determined if these proteins are critically required for *L. intracellularis* infection of enterocytes.

An antigenic and functional study was performed on the flagellin-like protein (LI0570) that was recently identified using *in silico* computational approaches (Won and Lee 2018). The recombinant LI0570 protein was detected in Western blots using mouse anti- *L. intracellularis* hyperimmune sera indicating that this protein is immunogenic (Won and Lee 2018). Further, HEK-Blue cells incubated with the rLI0570 protein led to stimulation of TLR-5 signaling and IL-8 production which implies its function in innate immune cell, *in-vivo* (Won and Lee 2018). Due to the potential immune-stimulatory effect of rLI0570, this recombinant protein has a dual role as antigen and adjuvant and may be an excellent candidate to develop recombinant subunit vaccine. Currently proteins which comprise the Type III secretion system (T3SS), autotransporter protein (LatA), LI0841, and LI0902 were characterized and shown to have immunogenic properties (Watson et al. 2014; Watson et al. 2011; Won and Lee 2018). Other uncharacterized bacterial proteins that are localized to the surface localization and that play a role in infection are potentially important immunogens

As an alternative approach to identify *L. intracellularis* antigens, we used functional analysis coupled with two-dimensional gel electrophoresis (2DE), Western blot analysis and mass spectrometry to help select the antigens that are targeted by the humoral immune system. Bacterial components and mechanism that facilitate attachment of bacteria to enterocytes has not yet been fully determined but they are possible facilitators for invasion of intestinal epithelial cells and are therefore potential immunogens (Vannucci and Gebhart 2014). We sought to identify these proteins. Briefly, we lysed the avirulent *L. intracellularis* from the Enterisol vaccine and labeled the proteins present with Cy5- dye (Obradovic et al. 2019). These labeled proteins were then incubated with plated but undifferentiated porcine intestinal epithelial cells (IPEC-1). The cells were rinsed then ruptured and the IPEC-1 proteins and adherent Cy5-labeled L. intracellularis proteins were subjected to 2DE. The proteins were then transferred to a nitrocellulose membrane and probed with serum from rabbits that were vaccinated with Enterisol plus Incomplete Freund's Adjuvant adjuvant multiple times. The *L. intracellularis* proteins that were bound by the rabbit antibodies are antigens of interest and they were sent for mass spectrometry for analysis. We identified 11 putative antigens (Obradovic et al. 2019) and cloned 6 of them: Flagellin (fliC, LI0710), Putative outer protein N (LI1153), ABC dipeptide transport system (LI0169) and autotransporter (LI0649), LatA. Among the 11 proteins detected in this study, proteins Chaperonin GroeL (LI0625) and 5'-nucleotidase/2', 3' cyclic phosphodiesterase (LI1171) were also reported previously using a shot-gun proteomic approach (Watson et al. 2014). The genes for these select proteins were cloned into plasmids, expressed in E. coli and purified. Western blot analysis was performed using both rabbit serum from Enterisol vaccinated rabbits as well as serum from pig barns that had been infected with pathogenic *L. intracellularis* to confirm that these recombinant proteins were immunogenic. Flow cytometric analysis on select antigens confirmed that neutralizing antibodies reduced the ability of L. intracellularis to invade IPEC-1 cells (Obradovic et al. 2019).

To establish whether these recombinant proteins were immunogenic, we performed several vaccine trials in pig using multiple vaccine adjuvants. We immunized pigs with a trivalent vaccine (FOG vaccine consisting of rFLiC, rOppA protein (a ABC Type dipeptide transport system) and rGroEL (a stress response protein)) and a divalent vaccine (CM vaccine consisting of rClpP (an ATP-dependent Clp protease proteolytic subunit) and rMetK (a S-adenosyl methionine synthase)) all formulated with the oil-in-water adjuvant Emulsigen® (Fourie et al. 2021). Relative to the control pigs, pigs immunized with the FOG vaccine produced robust and significantly higher serum IgG antibodies against rFliC and rGroEL, and significantly higher anti-FliC and anti-GroEL IgA antibodies in jejunal (GroEL only) and ileal intestinal mucosa (Fourie et al. 2021). Pigs immunized with CM vaccine produced significantly higher serum antibodies against rClpP and rMetK and significantly higher anti-rClpP IgA antibodies in the ileum relative to the control pigs. Quantitative polymerase chain reaction (qPCR) analysis showed that 18 days after challenge with infectious *L. intracellularis,* challenged/control pigs and pigs that received the CM vaccine, but not the pigs vaccinated with the FOG vaccine, shed significantly more bacteria in feces than the unchallenged controls pigs. These data suggest that the FOG vaccinated pigs showed some, albeit limited, protection. These data suggest that antigens FliC, GroEL and ClpP should be investigated further using different adjuvants to promote robust humoral and cell-mediated immunity.

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Subunit T cell antigen selection and analysis

Whether pigs are infected or vaccinated prior to re-exposure can impact the cell-mediated immune responses observed. Following primary infection of 5-6 week old piglets, some piglets were reinfected and some were vaccinated. The IFNγ responses to the initial infection were moderate with around 50% of the pigs responding with Ag-specific IFNγ above background level (100 pg/mL), and with several pigs showing a sustained high Ag-specific IFNγ response even at day 48 pi (Cordes et al. 2012). After rechallenge (RE), the RE pigs showed a memory recall cell-mediated immune response which was significantly stronger compared to the primary response in age-matched challenge control (CC group) pigs as assessed by whole blood IFNγ assay and by flow cytometry. The major IFNγ producing cells were identified as CD8+ and CD4+CD8+ double positive lymphocytes. However, when a similar trial was undertaken comparing the CMI response between re-infected group (RE group) and Enterisol-vaccinated group (VACC group), a difference in immune response and protection was observed (Riber et al. 2015). The VACC group showed antigen-specific proliferation at background levels after challenge, whereas the RE-pigs at 2nd challenge showed significantly increased responses, especially in the $CD4^+CD8^+$ T cell population, peaking at day 18 or 26 post challenge with a decline at day 33 post challenge, compared to VAC-group, which in turn showed responses comparable with the responses in the non-vaccinated CC-group. Only the RE-challenged pigs were fully protected against clinical disease suggesting that mediators of protective immunity against *L. intracellularis* were likely CD8+ effector cells and CD4+CD8+ double positive memory T cells (Riber et al. 2015).

Identification of T cell epitopes from the entire bacterial or viral proteome is a very challenging field. We have devised a functional assay wherein we use extensive gel electrophoresis of the entire L. intracellularis proteome to test which proteins are responsible for induction of the T cell-mediated IFNg secretion from PBMCs from LI-challenged and/or vaccinated pigs (manuscript in preparation). For our identification of T cell epitopes, we have ensured sufficient number of memory T cells in the PBMC population and an effective protein purification method that reverses denatured proteins back to biological active form in a low cytotoxic buffer. Further, we have performed extensive immunoinformatic analysis to identify T cell epitopes that will then be employing reverse vaccination. From a pool of ~ 1350 recorded *L. intracellularis* proteins, we focused on known expressed proteins in a pathogenic strain as well as proteins with unknown functions. Next, entries were selected based on antigenicity at ≥ 0.5 thresholds out of 1 which narrowed the number of proteins down to approximately 400. The solubility, high content of transmembrane helices, toxicities and physio-chemical properties of selected proteins further narrow down the selection. The analyses help us to exclude more proteins from the list due to having largely nonpolar contents and low solubility, in other cases, they compose 20% in minimum for transmembrane segments or toxicity observation danger enough to interfere with the piglets' metabolism. The remaining candidates were separately used as an input for Immune Epitope Database Analysis Resource (IEDB) and NetMHCpan 4.1 servers to be processed and for obtaining epitopes bound to piglets' swine leukocyte antigen (SLA) receptors. The higher ranked epitopes were extracted for evaluating their antigenicity, toxicities and to analyze their protease sensitivities. Among all epitopes, the selected candidates demonstrated predicted resistance to degradation from a diverse range of proteinases e.g., trypsin, chymotrypsin, and many others. All chosen epitopes were then used as inputs for homology modelling providing three-D conformations and docking tools producing SLA - epitopes binding poses to further be analyzed by means of Molecular Dynamics (MD) simulations. Twenty-eight epitopes were finalized for conducting MD simulations of SLA receptors with their docked epitopes. We performed 300 nanoseconds of MD production run and included the following criteria: stability, solvent-surface accessibility, radius of gyration and binding free energy between each epitope and its SLA. The epitopes with large negative free energy ≥ - 20 kJ/mol and the best conformational stability were the end-point selections for generating the multiepitope vaccine construct (manuscript in preparation). A synthetic gene coding for the selected epitopes are being developed and the corresponding proteins will be tested for antigenicity in a subunit vaccine in pigs.

Conclusions

Overall, we predict that a subunit vaccine would be a very attractive option for producers because comparative analysis of antibody response can be used to discern whether the animal has been vaccinated or infected. Once antigens combined with appropriate adjuvants are selected to stimulate neutralizing antibodies and/or robust CD8+ and CD4+CD8+ T cell activation, we predict reliable protection against disease. Taken together, these data indicate that ideal vaccine should be able to elicit balanced immune response where both cell-mediated and local humoral immunity work in concert to eliminate *L. intracellularis*-associated disease.

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Changing a dogma: What is the impact of systemic immunization on the gut and respiratory mucosal immune response?

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Diseases that affect pig's life and, consequently, the pig production system, are caused mostly by microorganisms that use the mucosa as a front door. From the interaction with the mucosa, some pathogens produce local inflammation while others travel to more distant sites and cause, for example, arthritis and meningitis. Therefore, the modulation of immunity associated with pathogens gateway is an intelligent strategy to control infections and, consequently, to prevent the development of diseases.

Modulation of the mucosal-associated adaptive immunity might be achieved through natural infections or by the use of vaccines. Regarding the former, unfortunately, in most cases, upon encounter with the pathogen the animal first develops the disease and during this process generates humoral and cellular responses that fight the disease-causing agent. In practice, this is not interesting because disease outbreaks are associated with high economic losses and, in the context of pig production, they can reach significant proportions due to the large number of animals confined in the same facility. With regard to vaccines, the dynamic of the process is completely different in comparison to natural infection; during vaccination, a biological product is used to stimulate the development of a functional adaptive immune response without any associated disease. However, the success of vaccine prevention is conditioned to: i) type of vaccine; ii) correct timing of vaccine application; iii) the animal's immunological status and iv) the absence of some antibiotics during the genesis of the adaptive response.

Due to the high frequency of mucosal contact with foreign substances (through ingestion or by inhalation), during the primary development of the immune system, cellular strategies to tolerate food, environmental antigens, and the microorganisms that form the different microbiota were evolutionarily developed; thus, breaking this tolerance through artificial strategies (e.g. vaccination) is necessary when it is desired to induce local immunity by the delivery of antigens via the mucosa route.

Although the mucous membranes have different primary functions (e.g. respiratory versus enteric), they have a similar and tightly connected immunological organization. In the respiratory and enteric mucosa, it is possible to find different classes of antibodies (SIgA, SIgM and IgG) (Chen et al., 2020) and antimicrobial peptides (Saeed et al., 2022) immobilized by mucin on epithelial cells. Intraepithelial lymphocytes (CD2$^+$CD8α$^+$ γδ and CD4$^-$CD8α$^+$ αβ T cells) (Piet et al., 2011; Wiarda et al., 2020), immunoglobulin-secreting B lymphocytes and effector T cells present in the lamina propria (Bailey et al., 1998; Brandtzaeg and Johansen, 2005), macrophages and dendritic cells, present at the sites of immune response induction, are responsible for defending the mucosa from infections caused by pathogenic microorganisms, and for immune-controlling the microorganisms that make up the microbiota.

Over the last few decades, we have learned that IgA is the main immunoglobulin class present in the mucosa, and we heard very little about the implication of other classes, such as IgM and IgG, in the control of mucosal microorganisms. IgA is mainly produced by mucosa-associated B cells in response to stimulation by commensal microorganisms; in this case, antibody production (polyreactive secretory IgA - SIgA) occurs without the involvement of T helper lymphocytes (T cell independent pathway). In this pathway other factors such as TLR ligands, innate signals from the gut microbiota, dendritic cells and epithelial cells, can directly stimulate the activation of B cells (Cerutti, 2008; Fagarasan et al., 2010; Gutzeit et al., 2014). On the other hand, when invasive microorganisms cause mucosal infections, B lymphocytes mount IgA responses after to be stimulated by helper T lymphocytes (T cell dependent pathway) (Cerutti, 2008; Fagarasan et al., 2010; Macpherson et al., 2018), which leads to the formation of a germinal center and production of highly specific IgA.

At mucosa, SIgA plays an important role in the neutralization of pathogenic and commensal microorganisms; this process is called immune exclusion (IE) (**Figure 1**). The IE is important to reduce the pro-inflammatory stimuli generated by resident microorganisms, and SIgA does this task by preventing the physical contact of surface molecules of the pathogens with the pattern recognition receptors expressed on epithelial or immunological cells associated with the mucosa. Although SIgA does not activate the classical pathway of the complement system, SIgA coated-microorganisms that reach the lamina propria can be recognized by pro-inflammatory phagocytes that express the Fcα R receptor on their surface; in this case, inflammatory responses are generated (Breedveld and van Egmond, 2019; Monteiro and Van De Winkel, 2003; Pasquier et al., 2005).

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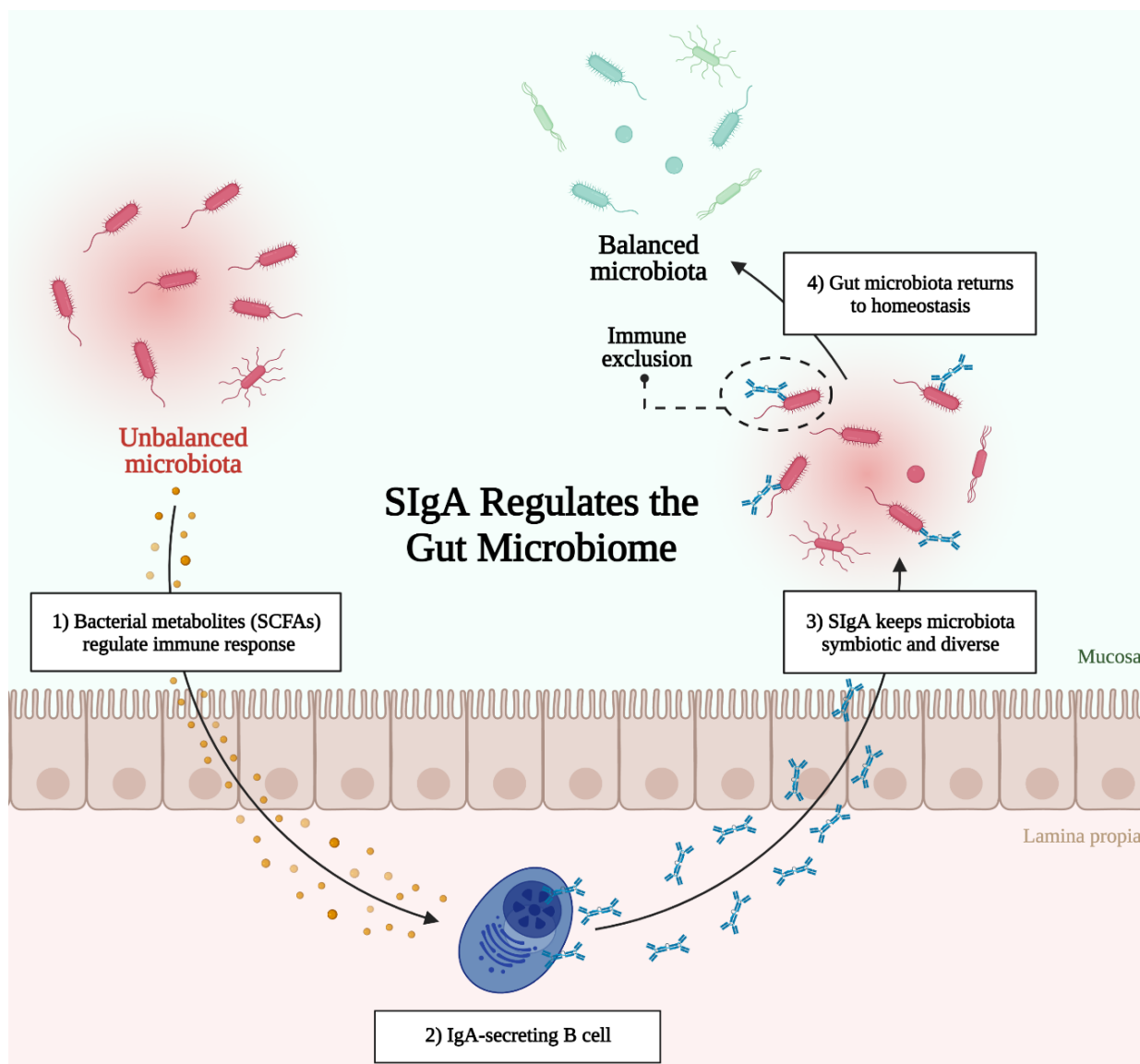
Figure 1. Impact of gut metabolites on gut IgA responses. Short-chain fatty acids (SCFAs, e.g. butyrate and propionate) augment gut SIgA (secretory IgA) production by modulating B cell response. SIgA mediates immune exclusion, which exclude less beneficial microorganisms. Additionally, SIgA quenches the motility, growth and pro-inflammatory properties of commensals, regulates their metabolic output and promotes their sampling by M cells (Chen et al., 2020). Figure adapted from "IgA Role in Maintaining Colonic Homeostasis", by BioRender.com (2022).

Another class of antibody found in the mucosa is IgM, which is the most ancient member of mammalian antibody family (Flajnik, 2002). In general, IgM generates the first line of humoral defense against pathogens (Chen et al., 2020) and its primary biological function is the activation of the complement system which is an important mechanism for microbial clearance and initiation of inflammation (Ehrenstein and Notley, 2010). Albeit IgM is more abundant in the blood, and structurally large, it can also rich mucosal surface as secretory IgM (SIgM) (Brandtzaeg and Johansen, 2005).

Mucosal IgG is one of the most abundant classes of antibodies present in the lower respiratory tract, with titers equal to or even greater than SIgA (Brandtzaeg et al., 2001; Brandtzaeg et al., 1999). On the other hand, in the upper respiratory tract and in the enteric mucosa, IgG levels may be lower when compared to SIgA; however, upon an infectious stimulus the IgG levels may become higher than those of IgA (Bjerke and Brandtzaeg, 1990; Bjerke et al., 1986; Brandtzaeg et al., 2001; Brandtzaeg and Johansen, 2005; Macpherson et al., 2008). In our laboratory, in controlled immunization studies, we observed that some vaccines stimulate higher levels of IgG in relation to SIgA at the upper respiratory and enteric mucosa; and, some vaccines, on the other hand, only stimulate the production of SIgA on the upper respiratory mucosa. In the lumen of mucosa, monomeric IgG likely exerts its homeostatic and defensive functions in cooperation with polymeric SIgA and SIgM (Chen et al., 2020). In pigs, two IgG subclasses are mainly produced and they have differences in their biological functions. IgG1 has less ability to activate the classical pathway of the complement system compared to IgG2 (Crawley and Wilkie, 2003) and, therefore, vaccines

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that modulate the production of IgG2 might be better than those that induce predominantly IgG1. The highly protective value of IgG in the gut mucosa can be seen in mice infected with *Citrobacter rodentium*. Eradication of this attaching-and-effacing pathogen from the intestinal mucosa requires IgG, but not SIgA or SIgM (Bry and Brenner, 2004). During the defense, IgG selectively opsonizes virulent bacteria, which are subsequently killed by neutrophils that transmigrate from the lamina propria to the intestinal lumen (Bry and Brenner, 2004; Caballero-Flores et al., 2019; Kamada et al., 2015).

As illustrated in **Figure 2**, both dimeric IgA and pentameric IgMs are translocated from the lamina propria to the mucosal surface via the polymeric immunoglobulin receptor (pIgR). Intraluminal IgA and IgM, called secretory IgA and IgM (SIgA and SIgM) have in their structure a peptide fragment derived from pIgR, called the secretory (S) component. This peptide increases the survival of antibodies by avoiding proteolytic degradation by intestinal enzymes. On the other hand, IgG is translocated to mucosa through the FcRn receptor, which bidirectionally transports IgG across epithelial cells from intestinal, pulmonary, genital and mammary mucosae (Rath et al., 2014).
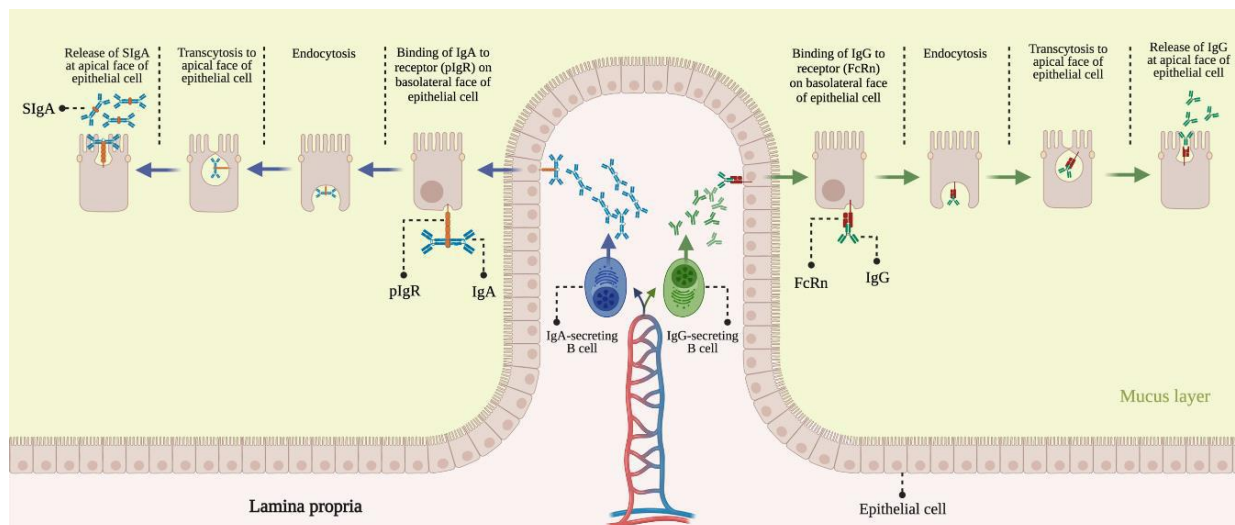
Figure 2. Immunoglobulin translocation across the mucosa membrane. Dimeric IgA and pentameric IgM are translocated from lamina propria to mucus layer via the polymeric immunoglobulin receptor (pIgR) (left-side), while IgG are translocated via the neonatal Fc receptor (FcRn) (right-side). Figure created with BioRender.com (2022).

Over the last 10 years, our research group has been working on the development and evaluation of vaccines against *Glaesserella parasuis*, the etiologic agent of Glässer's disease. Although protection from infection is the primary goal of most vaccine development programs and is the feature that most regulatory agencies are looking for, the ability to prevent or eliminate colonization is a much more effective preventative measure. In a recent study conducted by our group we demonstrated that immunization with a vaccine preparation containing the TbpB^{Y167A} protein not only prevented infection by *G. parasuis* but also eliminated the natural colonization by this bacterium in the pig (Frandoloso et al., 2020).

In that study, we observed that regardless of the application route (oral intraepithelial, intradermal, intramuscular or the combination of oral intraepithelial and intradermal routes) of the vaccines, all of them stimulated the production and translocation of SIgA, IgG1 and IgG2 to the respiratory mucosa (Frandoloso et al., 2020); these finding directly demonstrate that systemic routes of immunization can stimulate mucosal immunity.

Interestingly, and contrary to what was described by other authors when analyzing other vaccines based on distinct antigens, the levels of mucosal IgGs induced by vaccines based on the TbpB^{Y167A} protein were significantly higher than those of IgA. Biologically, this immunological feature is very interesting in the defense against *G. parasuis* since this pathogen, during infection, secretes a protease that specifically cleaves SIgA but not IgG. Therefore, the presence of functional IgGs at the site of entrance of *G. parasuis* can prevent respiratory colonization, as demonstrated by our group (Frandoloso et al., 2020). In this line, and because we observed that the type of vaccine formulation (microparticles versus classical subunit) and the vaccine application site generated IgG responses with different capacities to prevent *G. parasuis* colonization, we are deepening our investigations into the characteristics of mucosal IgGs capable of controlling the colonization of this pathogen.

In order to understand how our vaccine based on the TbpB^{Y167A} protein could protect pigs challenged with lethal doses of *G. parasuis* SV5 and SV7, we conducted several *in vivo* and *in vitro* studies (Barasuol et al., 2017; Frandoloso et. al., 2020; Frandoloso et al., 2015; Guizzo et al., 2018). As illustrated in **Figure 3**, the protective mechanism provided

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by the TbpB^{Y167A}-based vaccine is entirely humoral. Briefly, immunized pigs (two-dose vaccine protocol) produced high titers of systemic and mucosal IgGs capable of: (a) blocking the interaction between *G. parasuis* TbpB and porcine transferrin which restricts the iron uptake, and consequently, *G. parasuis* depletes its iron reserves and cannot replicate (**Figure 3A**); (b) efficiently activating the classical pathway of the complement system (**Figure 3B**) which is a powerful weapon to killing *G. parasuis*; and (c) efficiently opsonizing *G. parasuis,* increasing the efficiency and speed of phagocytosis (**Figure 2C**). Altogether, these results provide strong evidence that a mutant TbpB vaccine preparation could be developed to eliminate *G. parasuis* from commercial pig barns.
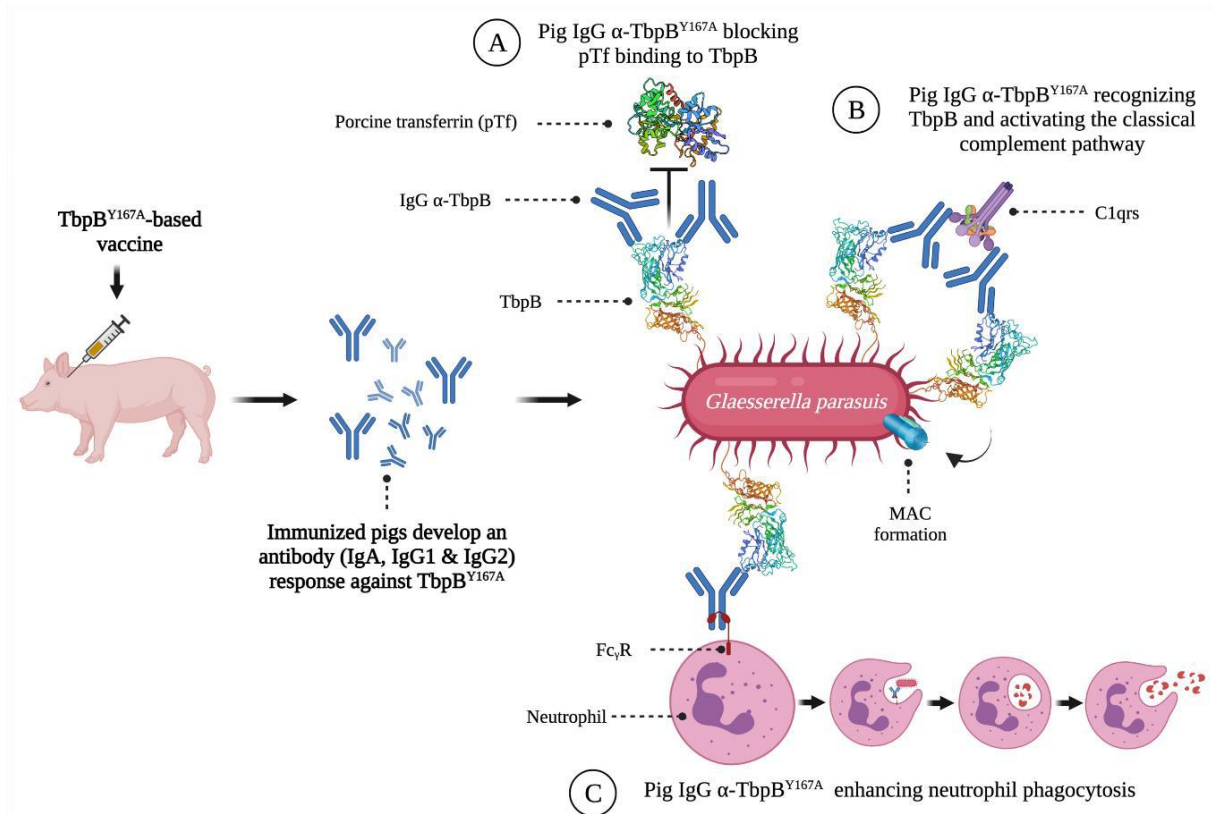
Figure 3. Illustration of the Immunobiological mechanisms mediated by anti-TbpB IgGs. A) Neutralization of the iron uptake receptor by IgG anti-TbpB. B) Activation of the complement system via the classical pathway. C) Opsonophagocytosis. All these mechanisms have been demonstrated in our previous studies (Barasuol et al., 2017; Frandoloso et al., 2020; Frandoloso et al., 2015; Guizzo et al., 2018). Figure created with BioRender.com (2022).

In parallel with the vaccinology studies on *G. parasuis*, we started the characterization of the immune response of two licensed vaccines against *Lawsonia intracellularis*, the etiological agent of Porcine Proliferative Enteropathy. In this study, using specific pathogen free pigs as animal model, we observed that both the inactivated-vaccine, administered by the intramuscular route, and the live-attenuated vaccine, administered orally, induced SIgA and IgG on the ileum mucosa. Interestingly, the sera IgG1 and IgG2 titers induced by the intramuscular vaccine were statistically ($p<0.0001$) superior to those induced by the live-attenuated vaccine. Additionally, the intramuscular vaccine induced higher levels of IgG2, while the live attenuated vaccine modulated a sharper production of IgG1. The same biological trend was observed at ileum mucosa; but the level of total IgG induced was not different between the two vaccines (Frandoloso et al., *manuscript in preparation*).

These results show that the adaptive humoral response associated with mucosa is much richer than we previously thought. Elucidating the role that IgG plays in controlling pathogens at the point of entry will help us to design more effective vaccines, which can prevent the colonization of specific pathogens and, consequently, eradicate diseases that affect the life and production of swine.

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Immunonutrients and their applications in swine production

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In the intestines, the immune system is interconnected with cells with digestory functions, as well as with neurons that coregulate both digestion and immunity. The three cell types – immune, neuronal and digestory – sense and respond to the ingesta transiting in the intestinal lumen. Thus, ingested substances can regulate the function of such cells, via direct mechanisms (such as by providing nutrition or by activating cell receptors) or indirect mechanisms – immune cells can sense the condition of altered enterocytes, for instance, or can respond to neuronal activity. Whereas clinical or even subclinical malnourishment is rare in modern swine production systems, nutritional guides were created having maximal productivity in mind, albeit not considering for immune activity. Animal diets are often studied and determined in optimal sanitary conditions, therefore not reflecting the common nutritional demands that are poised by infection. Optimal performance of immune cells requires a combination of nutrients and of other feed substances that are different from those required for muscle cell growth, for instance. Intestinal neurons and enterocytes will also signal to immune cells during "challenge", and this has rarely been assessed during feed formulation. Here we will discuss several nutrients and other feed components that can influence on cells within the intestine which can alter the function of mucosal immunity.

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Nutritional means to facilitate the post-weaning transition of piglets without antimicrobials

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Introduction

Antimicrobial compounds including antibiotics and mineral compounds, e.g., ZnO and CuSO4, are effective management strategies to assist in overcoming the post-weaning transition and preventing, controlling and (or) treating infectious enteric diseases. However, times have changed, antimicrobial resistance (AMR) is a key issue (Aarestrup et al. 2008), and the restricted use, or no use at all, of antimicrobials is increasingly on the agenda worldwide for pork production (Bonetti et al. 2021). Producers can adopt best practices in biosecurity, health care, animal welfare, genetics, farm management, staff training, feed handling and animal nutrition to the extent that is possible from a practical and economical perspective, in order to overcome these issues. In general, such measures focus on reducing infection pressure in the environment and increasing the animals' disease resistance and resilience. Minimizing stress, both social and environmental, well-targeted tailor-made vaccination schemes and, last but not least, 'health-promoting' diets fed in the post-weaning period, will assist in overcoming the post-weaning malaise (Smits et al. 2021).

With respect to animal nutrition, there is a clear association between a 'healthy' gastrointestinal tract (GIT), that is, one that functions in an optimum manner in the post-weaning period, and the nutrition the piglet receives. This may or may not be causal, but nonetheless, disruptions in the GIT after weaning that cause losses, for example, due to post-weaning diarrhea (PWD), can have a nutritional etiology that is most likely exacerbated in the absence of the antimicrobial compounds that were traditionally relied upon. It is important, therefore, to better understand the complex and interrelated mechanisms that affect the structure and function of the GIT in the post-weaning period, to support and optimize the GIT with non-antimicrobial solutions. In this regard, nutrition plays a vital role.

The post-weaning transition of piglets

There are many published papers and reviews related to the post-weaning transition of piglets and the issues and difficulties associated with their management following weaning (e.g., Hampson, 1994; Heo et al. 2013; Pluske et al. 2018). Post-weaning diarrhea remains one of the most economically relevant diseases in pork production due to the costs of therapies, slower growth and poorer feed efficiency, increased variation in weights at slaughter, and increased morbidity and mortality. This is accompanied by marked alterations in the architecture, enzymology, metabolism, immunology and biochemistry of the GIT, with the weaning process implicated in these changes (Pluske et al. 1997). As alluded to previously, antimicrobial compounds are commonly used to transition the piglet to the post-weaning production phase, but this is no longer possible in some parts of the world. Consequently, there is a plethora of published papers, articles, reports, podcasts and so on exploring the many in-feed and (or) in-water alternatives or replacements to the use of antimicrobial compounds and strategies to try and successfully transition piglets after weaning. Indeed, de Lange et al. (2010) observed at the time that a large amount of research had already been conducted evaluating the impact of a wide range of feed ingredients and feed additives on various aspects of GIT health and development in pigs, and such research and evaluation should continue to find the best possible combination of strategies that provide reliable and consistent responses in the field. The exploration continues to this day.

Nutritional strategies to assist with the post-weaning transition

A wide number of dietary and (or) water interventions and strategies are available to industry to ameliorate the post-weaning challenges, even when antimicrobial treatments can be used, or wish to be used less (antimicrobial stewardship). Such options are widely available to the pork industry and in some instances, commonly and successfully adopted already. Some countries, e.g., Sweden, have been managing pigs without the use of antibiotics, including in the nursery, for many years. Improvements in biosecurity, management, feeding and disease control have assisted in keeping overall therapeutic antibiotic use low, but nonetheless, antibiotic use on Swedish pig farms still varies considerably. Backhans et al. (2016) reported that factors influencing antimicrobial use in Swedish farrow-to-

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finish pig farms were related to individual farmer characteristics such as age, gender and years of experience more than production-related factors. Similarly, a report from SEGES in Denmark (https://www.pig-world.co.uk/features/pig-production-in-a-zinc-oxide-free-and-low-medication-world-the-danish-perspective.html) showed that from 26 pig farms that had already eliminated the use of medicinal (> 150 mg/kg) ZnO in weaner diets, the skill and experience of the staff were key factors in that success. In 20 out of 26 farms, the nursery manager had an employment period on that specific farm of more than 12 months. In 18 out of 26 farms, the nursery manager had worked with piglets for more than 3 years. Moreover, all farms except for two had taken special measures to increase feed intake after weaning, e.g., by extra feeding on the floor and additional feeding spaces in troughs, and an extra drinking space. Increased feed intake after weaning is well recognized as a risk factor for reducing PWD (Pluske et al., 1997). Therefore, special and diligent attention to management in the nursery is essential for a successful weaning transition without the use of antimicrobial compounds such as ZnO.

Be this as it may, management through the diet and (or) water to assist piglets with weaning, prevent enteric infections and disorders such as PWD, and maintain 'gut health', is simply more attractive to many producers, particularly in times when labor is constrained. Given the GIT of the weaned piglet is undergoing rapid structural and functional changes during this time, it is unlikely that a single dietary-related strategy can be effective, and uniformly, in optimizing 'gut health' given the wide-ranging conditions of housing, management, feeding and health status that pigs are grown under. Therefore, direct substitution of an antimicrobial compound such as an antibiotic or ZnO for a feed additive, or a diet-related strategy such as lowered protein content, to accomplish the same positive effects after weaning, is unlikely (Pluske, 2013; Pluske et al. 2018). This emphasizes the need to explore underlying mechanisms when evaluating the functional properties of feed ingredients and feed additives, so that a better understanding can occur to achieve the optimal response to dietary interventions. As the production benefits from feeding antimicrobials is achieved through many different mechanisms, likewise the strategy for replacing them will depend on a combination of nutritional, management, housing, health and (or) husbandry factors (Pluske et al. 2002; Pluske, 2013).

It is not the intention in this paper to describe the array of dietary and (or) water strategies that exist for the industry. This is reflected in the number of peer-reviewed papers searchable in PubMed (using intestinal health in pigs as keywords in the title or abstract). Since 1960 and until 2005, there were <10 papers searched in PubMed, which had increased 10-fold by 2018, and then having 180 papers in 2020 (Zheng et al. 2021). However, and with regard to feed additives, there is often scepticism on how effective these feed additives are and if they can fully replace antimicrobials after weaning. One objective methodology to try and evaluate the use of potential dietary feed additives having antibacterial effects on the performance of weaned piglets is through meta-analysis. Vanrolleghem et al. (2019) examined 23 peer-reviewed *in vivo* studies, comprising 50 trials, in which the dietary feed additives were grouped into five classes: antimicrobial peptides, chitosan, lysozyme, medium-chain fatty acids/triglycerides, and plant extracts. The authors' reported that for each class of dietary feed additives, results showed significantly higher averagedaily gain in the group with dietary feed additives compared to the negative control group, while no significant difference with the positive control group (antimicrobial compound) was seen. Furthermore, a positive effect on FCRwas found in the group with dietary feed additives compared to the negative control group. No significant differenceswith positive control groups were observed for each class of dietary feed additives, except for plant extracts, where the FCR was also significantly reduced in the treatment group.

Of course, there are many other feed additives and dietary strategies available for use in the industry to modify different aspects of 'gut health' after weaning that were not assessed in the review by Vanrolleghem et al. (2019), forexample probiotics, prebiotics, dietary fibers, enzymes, immunoglobulins, and protein content of the diet (de Lange et al. 2010; Pluske et al. 2018). There are also studies that show opposing effects, or no effects at all, on indices of 'gut health' and production in the post-weaning period. Extensive studies have also been conducted previously (e.g., described in Heo et al. 2013) into the roles that protein content of the diet plays in PWD, and it is now clear that reducing the overall protein content of the diet fed to pigs after weaning can reduce PWD, compared to a positive control group such as ZnO. However, some studies demonstrate a concomitant reduction in production after weaningwith feeding a lower protein diet, and in circumstances where antimicrobials such as ZnO or antibiotics can still be used, this strategy is not warranted. In such cases, other additives in the diet can restore this production drop, but usually at a higher cost of production.

Can piglets be weaned without any medications?

A matter of discussion arising in relation to these issues is whether, or not, it is possible to successfully negotiate the post-weaning transition without the use of any antimicrobial compounds at all. Such an issue needs to be considered in the overall context that antibiotics are an important part of health programs, since ensuring the health and well-being of animals is both an ethical obligation and a critical component of providing safe food products (Singer et al. 2019). Nevertheless, and because of the high public awareness of antibiotic use and AMR in some countries, as well as the opportunity to establish a market niche, production of pigs without antibiotics ("Raised Without Antibiotics";

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RWA) is now practiced in some systems. A study in Denmark by Lynegaard et al. (2021) used a total of 518 pigs in herd A and 436 pigs in herd B, individually ear-tagged and subjected to weekly investigations of antibiotic treatment status from birth to 12 weeks of age, to explore risk factors for antibiotic treatment and investigate growth performance of pigs. Lynegaard et al. (2021) reported that at 12 weeks of age, the number of pigs treated with antibiotics was (unsurprisingly) different between the two herds, and individual pigs that required antibiotic treatment had a reduced average daily gain from day 0 to 28 in both herds. Additionally, significant risk factors for antibiotic treatment were identified as a low bodyweight in herd A, whereas barrows and litters with less than 19 piglets were the main risk factors in herd B.

In production systems where nursery pigs are raised under antibiotic-free conditions (RWA), nutritional considerations are also required. A key consideration is the amino acid profile and content of the diets. Total sulfur amino acids play a critical role in numerous biological functions, including antioxidative status and immunity, as well as protein synthesis. Ren et al. (2021) investigated the effects of increasing the standardized ileal digestible (SID) total sulfur amino acid to lysine (TSAA:Lys) on the growth performance of nursery pigs raised with or without antibiotics, and to determine the optimal SID TSAA:Lys in nursery pigs raised without antibiotics (50 mg/kg carbodox). Results from the two experiments showed that SID TSAA to Lys requirements under an antibiotic-free feeding regime in the first 21 days after weaning were 62% and 72% in terms of average daily gain and gain:feed, respectively, whereas an SID TSAA:Lys of approximately 58% was required to maximize average daily gain and gain:feed for the late nursery phase. These data concur with those of Capozzalo et al. (2017) using an enterotoxigenic *E. coli* model as a challenge under antimicrobial-free conditions, and indicate that nursery pigs fed diets containing no antibiotic had a greater requirement of SID TSAA:Lys, this being ~ 5% higher than the NRC (2012) recommendation, than those fed diets containing an antibiotic.

Conclusions

Nutrition is one of the pillars of GIT health and contributes to minimizing antimicrobial use in pigs, especially after weaning. The post-weaning growth check and enteric diseases including diarrhea are important contributors to overall productivity and continue to represent a major source of economic loss in some circumstances. Related to continuing concerns over the link between antibiotic-resistant strains of bacteria and the use of antimicrobials, a considerable body of research and investigation into the specialized use of feed ingredients, feed additives, feed form and (or) dietary strategies has occurred to transition the industry away from antimicrobials, where required. To minimize production and economic consequences associated with the removal of antimicrobials from diets, the on-going examination for effective alternatives/replacements is crucial. In parallel to nutrition, other strategies and factors including management (e.g., reducing stress at weaning), manipulation of weaning age and (or) vaccination strategies can also play roles in managing the weaning transition to a reduction in antimicrobial use.

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Improving pigs' resilience and adaptation to sanitary challenges via nutrition: a focus on energy and amino acids

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Introduction

Resilience can be defined as animals' ability to be minimally affected by physical, social, environmental and health challenges that might occur during its productive life and quickly return to the physiological and performance states that pertained before exposure to a disturbance (Laghouaouta et al., 2021; Colditz & Hine 2016).

Over the last years, this trait has been of utmost importance since animal production has become increasingly challenged by health and sanitary issues, decreased use of in-feed antimicrobial compounds, climate changes that includes heat stress and extreme weather conditions, and reduced adaptation capacity of modern genotypes. Indeed, intensive genetic selection for production traits (growth rate, feed efficiency and reproductive capacity) has led to impaired fitness and reduced environmental flexibility of production animals (Misztal, 2017). This decrease partially due to a greater fraction of energy and biological process spent on production process with a corresponding lesser fraction spent on other vital functions such as immune and thermoregulatory responses (Rauw, 2012; Dumont et al., 2014).

Global warming benefits the development and dissemination of disease vectors, parasites and pathogens in the environment. Production animals will then be more and more exposed to pathogenic parasites, virus and bacteria in the coming decades (Kimaro & Chibinga, 2013). Such changes will be accentuated in tropical regions where the association of high temperature and relative humidity create even more favorable conditions for vectors and pathogens development (Campos et al., 2017). Therefore, livestock production intensification in hot climate areas will lead to increased exposure of animals to sanitary challenges (Perry et al., 2013; Skuce et al., 2013).

When exposed to health challenges (bacteria, viruses, parasites, mycotoxins, poor hygiene conditions), defense mechanisms to maintain homeostasis and integrity of the organism are activated with negative consequences for the productive and reproductive performance (Campos et al., 2017). Defense mechanisms correspond to behavioral, physiological and immunological responses that are characterized by innate responses that provide defense of low specificity, and acquired responses that exhibit higher specificity for particular eliciting agents. The magnitude of the immune response is variable and influenced by the nature of the stimulus, the immune status and the nutritional condition of the animal. Such adjustments include fever, increased metabolic rate for activation and maintenance of immune and non-immune responses, and redistribution of nutrients from growth and production processes to support the inflammatory and immune responses (Figure 1).

Besides, sick animals are often in anorexic state and then must rely on body reserves mobilization (lipolysis of adipose tissue and proteolysis of skeletal muscle) to meet their nutritional needs (Le Floc'h et al., 2004). For instance, skeletal muscle catabolism contributes to increased circulating levels of amino acids which, in turn, can is used in the liver for the synthesis of acute phase proteins (and other defense cells in the body), as well as a substrate for gluconeogenesis (Le Floc'h et al., 2004).
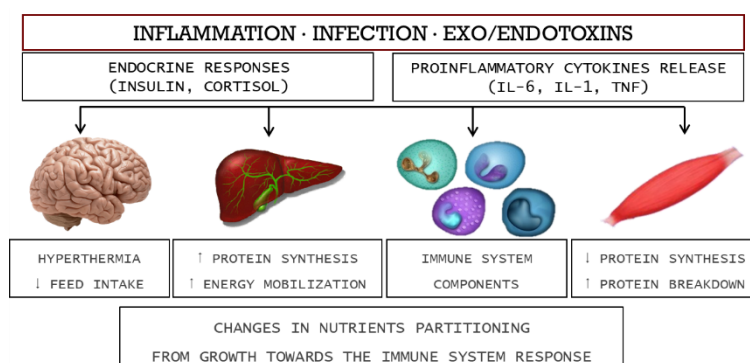

Figure 1. Immune system response and effects on nutrients utilization. Adapted from Campos et al. (2014)

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Among the strategies to increase resilience and preserve performance of farm animals subjected to challenging environmental conditions, special attention have been paid on energy and amino acid supplementation. Amino acids are organic compounds absorbed and used by the body for the synthesis of proteins, enzymes, hormones, structural components. They are major energy substrates for the intestinal epithelial and other rapidly growing cells, key constituents of gut barrier proteins, and have direct regulatory action on inflammatory and immune processes, oxidative stress, microbiota balance (Liu et al., 2017; Le Floc'h et al., 2018; Chalvon-Demersay et al., 2021). Therefore, the objective of this work is to provide an overview of how nutrition could be used as a strategic toll to improve pigs' resilience to sanitary challenges with a focus on energy and amino acids supplementation.

Increasing pigs' resilience to sanitary challenges via nutrition. Energy metabolism and requirements

In response to diseases and sanitary challenges, changes in dietary nutrient metabolism, energy and amino acids requirements, and reduced growth performance are consistently observed (Pastorelli et al., 2012). These changes are mainly related to: increased metabolic rate to support the overall immune and non-immune responses and particularly the febrile response; lower nutrient availability as a result of reduced feed intake; altered priorities for nutrient utilization as a result of neuroendocrine adjustments that reduce the capacity of non-immune tissues to use nutrients; proliferation of immune cells and changes in the turnover of tissue and plasma proteins; and tissue damage and repair in response to pathogens but also inappropriate or excessive activity of the immune and inflammatory response (Campos et al., 2017).

Fever and feed intake depression are the most common clinical signs of infection and systemic inflammation and are an important component of non-specific acute phase response (Steiner et al., 2006). The fever response is beneficial in terms of stimulating the synthesis of acute-phase proteins, induction of cytoprotective factors (heat shock proteins and anti-oxidant enzymes), increased bactericidal activity of neutrophils and macrophages, and inhibition of bacterial growth (Netea et al., 2000; Lee et al., 2012). To date, a 1.5 to 5°C increase in core temperature is observed during infection in mammals (Hasday & Singh, 2000). In growing pigs, a 2°C increase in eye temperature was reported in lipopolysaccharide challenged pigs compared to those injected with saline (Rakhshandeh et al., 2010). Fever is the major cost of the immune response requiring approximately a 7 to 13% increase in basal metabolism for each degree Celsius of increase in body temperature (Collier et al. 2008). Accordingly, Campos et al (2019; Figure 2) reported a consistent increase in the internal temperature from 38,7 to 40°C in pigs receiving repeated administrations of *Escherichia coli* lipopolysaccharide with a corresponded increase in maintenance energy requirements equivalent to 10% of the basal metabolic rate. In accordance, van der Peet-Schwering et al. (2019) suggested a 10 to 30% increase in energy maintenance requirements due to systemic immune system activation by non-pathogenic challenges, and a 7 to 12% increase in response to sanitary challenges.
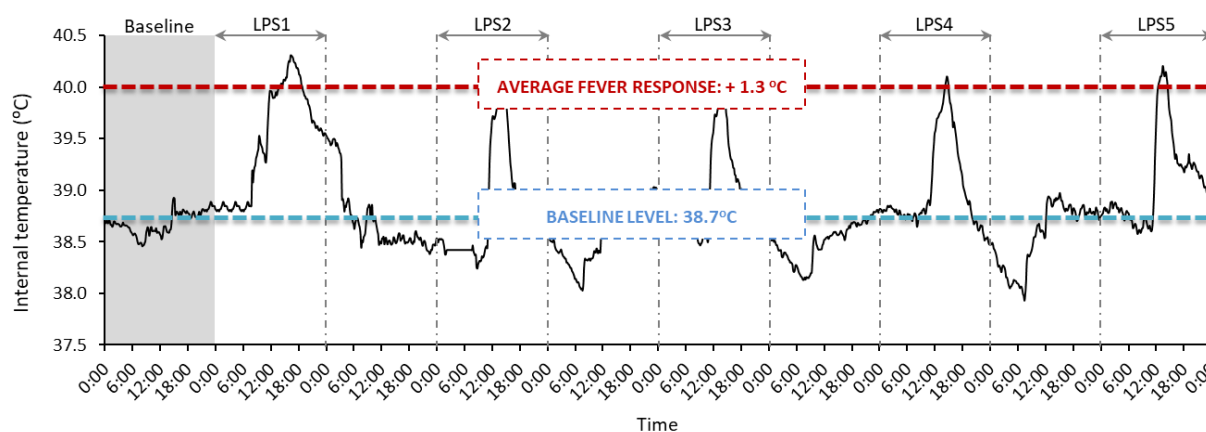
Figure 2. Kinetics of internal temperature of growing pigs before (baseline) and during a LPS challenge that consisted of five successive injections of LPS at 48 h intervals (Adapted from Campos et al., 2019)

Feed intake reduction in response to immune system activation results from pro-inflammatory cytokines action (Plata-Salaman, 1999, 2000; Konsman et al., 2002). Pro-inflammatory cytokines modulate gastrointestinal activities such as gastric emptying gut motility, and, ultimately, inhibit feeding. Cytokines can induce anorexia by a direct effect on specific neurons assumed to participate in the control of feeding. Furthermore, cytokines presumably stimulate the release of a group of hormones (glucagon, insulin and leptin) considered to be physiologic signals of satiety (Plata-Salaman, 2000).

The effects of different sanitary challenges on feed intake and growth in pigs were summarized by Pastorelli et al. (2012). Through a quantitative analysis of literature these authors reported significant reductions in feed intake in pigs

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exposed to different health challenges (systemic or gastrointestinal, live pathogens or non-pathogenic antigens). For example, a reduction in feed intake of about 8% was reported for digestive bacterial infections, 4% for poor hygiene housing conditions, 10% for LPS challenges, 23% for mycotoxicoses, 3% for parasitic infections and 16% for respiratory diseases. The reduction in feed intake is also observed in animals housed in challenging sanitary environmental conditions. Even at subclinical levels, whose consequences are not visually observed, Kyriazakis et al. (2008) observed a 75-80% reduction in feed intake in growing pigs.

Taking into account the increase in energy requirement due to the fever response and overall immune response, and the associated decrease in feed intake, a correspondent increase in dietary energy supply may be considered in order to improve pigs ability to perform under sanitary challenging conditions.

Protein and amino acids metabolism and requirements

As previously reported, when pigs are submitted to an immune challenge caused by infectious diseases, nutrients that might have been used to support growth are redirected to support the inflammatory response. In addition, sick animals are often in anorexic state and have to rely on body reserves mobilization to meet their nutritional needs. Therefore, skeletal muscle catabolism increases free amino acids into the circulation that, in turn, can be used in the liver for the synthesis of acute phase proteins as well as substrate for gluconeogenesis (Obled, 2003; Le Floch et al., 2018). It has also been suggested that protein catabolism during disease or inflammation is emphasized due to an imbalance between the supply of amino acids from endogenous proteolysis and those required for the synthesis of acute phase compounds (Reeds et al., 1994). Consequently, a greater amount of skeletal muscle protein might be degraded to provide amino acids to support the synthesis of equivalent acute phase protein (Johnson, 2012). Indeed, relative to muscle proteins, human acute phase proteins have greater amounts of aromatic (phenylalanine, tyrosine and tryptophan) and sulfur amino acids (cysteine; Reeds et al., 1994; Table 1).

Table 1. Partial amino acid profile of human skeletal muscle and acute phase proteins

	Skeletal muscle	Acute phase proteins (g/kg)				
		HP	CRP	Amyloid-A	α_1-Antitrypsin	Fibrinogen
Phenylalanine	40	30	105	103	83	46
Tyrosine	36	70	50	67	27	56
Tryptophan	13	32	42	45	11	35
Threonine	47	54	58	30	66	60
Cysteine	13	24	13	0	6	15

HP, haptoglobin; CRP, C-reactive protein. Adapted from Reeds et al. (1994).

Changes in plasma concentrations of some amino acids have been consistently observed in inflammatory-challenged pigs (Melchior et al., 2004; Campos et al., 2019; Fraga et al., 2021). In fasted pigs fed restricted meal, decreased plasma amino acids concentration in response to inflammatory and/or immune challenges could be associated with enhanced utilization to support the immune system. In growing pigs exposed to an inflammatory challenge caused by repeated administration of *Escherichia coli* lipopolysaccharide, a persistent decrease in postprandial concentrations of alanine, glycine, histidine, isoleucine, leucine, proline, serine, threonine, tryptophan and valine was observed evidencing a greater demand of these amino acids in such conditions (Campos et al., 2019). The increased requirements for branched-chain amino acids may be associated to their increased transamination and oxidation to be used as energy source for the immune function (Bruins et al., 2003). Increased threonine utilization is associated with the high content of this amino in acute phase proteins, such as haptoglobin (Reeds et al.,1994). Besides, threonine is one of the most abundant amino acids in immunoglobulins, and in mucins which are glycoproteins secreted in the intestinal lumen to protect the epithelium against injury (Le Floc'h et al., 2018). During intestinal inflammation, the gastrointestinal threonine utilization, immunoglobulins and mucins synthesis are increased reducing then threonine circulating levels and hence its availability to other tissues (Rémond et al., 2009). In regard to tryptophan, in addition to its high content in acute phase proteins, its catabolism is increased during inflammation through the indoleamine 2,3 dioxigenase pathway. For intance, Le Floc'h et al. (2008) reported increased indoleamine 2,3 dioxigenase activity in the lungs and associated lymph nodes in pigs suffering from chronic lung inflammation. Melchior et al. (2004) observed a permanent 10-days decrease in circulating levels of tryptophan in piglets suffering from chronic lung inflammation suggesting an increased clearance of this amino acid during the inflammation.

In agreement with the essential role of amino acids as precursors for the synthesis of bioactive peptides and low-molecular weight metabolites with major physiological and regulatory functions in animals (Le Floch et al., 2018),

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their functional supplementation has gained special attention. Functional amino acids are defined as those that regulate key metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of organisms or which form biologically active peptides or proteins (Wu, 2010). For example, in addition to the aforementioned amino acids, glutamine is important for a variety of metabolic processes participating in the synthesis of amino sugars and nucleotides and serving as substrate for immune cells (Le Floc'h et al., 2004; Li et al., 2007). Arginine is the precursor for the synthesis of nitric oxide by macrophages and neutrophils, which is an essential cytotoxic compound against invading pathogens. Additionally, arginine is also involved in the synthesis of polyamines, through the arginase pathway, that are particularly required for cell proliferation and differentiation (Reeds & Jahoor, 2001; Li et al., 2007). Bruins et al. (2003) reported increased alanine and glutamine uptake by the liver in association with increased synthesis of acute phase proteins in growing pigs challenged with lipopolysaccharides when compared to those receiving a saline solution.

In this context, the provision of specific free amino acids beyond the requirements for growth may contribute to a better ability (resilience) of pigs to respond and adapt to health challenges allowing then optimal performance, low mortality and good overall health. For instance, the use of functional amino acid to improve epithelial barrier function and absorption; intestinal immune fitness; oxidative stress homeostasis and microbiota balance has been recently reviewed by Chalvon-Demersay et al. (2021). In agreement, growing pigs housed in poor sanitary conditions had greater feed efficiency when fed diets with increased amino acids levels (+20% methionine, threonine, and tryptophan levels; van der Meer et al. 2016). Post-weaning piglets housed in poor hygiene conditions fed high tryptophan levels (+17%) had increased feed intake (Le Floc'h et al., 2009). Also, arginine, glutamine, glycine, and tryptophan are amino acids rapidly used in the gut where they regulate gene expression, cell signaling, antioxidative responses, and immunity. Therefore, their supplementation might be beneficial to overcome intestinal dysfunction during challenging periods such as weaning, poor sanitary condition, diets transition (Le Floch et al., 2018). More recently, the supplementation of methionine, threonine and tryptophan at above 20% of NRC (2021) improved growth performance and positively modulated the inflammatory response of weaned piglets orally inoculated with Salmonella Typhimurium (Rodrigues et al., 2021a,b). In close agreement with these findings, and in a recnt study, van der Peet-Schwering et al. (2019), reported that amino acids maintenance requirement for may increase with up 30% in pigs with an activated immune system.

Conclusions

Firstly, this works evidence that pigs exposure to health and/or sanitary challenges results in increased energy reequipments in association with fever and increase immune responses; and in increased demand and redistribution of amino acids to support the immune response. Secondly, it highlights the importance and effectiveness of supplementing amino acids based on their immune system function, that is: energy substrate, constituents of gut barrier proteins, regulatory action on inflammatory and immune processes, oxidative stress, microbiota balance. Finally, this information should be considered in nutritional programs to improve pigs' resilience to health and environmental challenges.

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Interactions of mycotoxins with the porcine immune system

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Introduction

Mycotoxins are toxic fungal secondary metabolites that contaminate agricultural commodities during cultivation, harvesting, transport, processing and storage (Bennett & Klich, 2003). Many filamentous fungi are toxigenic, and the most important producing genera are *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* (Pitt et al., 2000). Mycotoxins are present in a wide range of agricultural crops such as corn, wheat, rye, barley, rice, soybean, sorghum, nuts, and feed products (Marin et al., 2013), and recent estimations pointed out that 80% of agricultural crops contain mycotoxins (Eskola et al., 2020). Despite several prevention strategies on the field, good agricultural practices and adequate grain storage and transport, the presence of mycotoxins cannot be avoided.

To date, the most important mycotoxins from an occurrence and toxicological point of view are aflatoxins (AF) including aflatoxin B1 (AFB1), deoxynivalenol (DON), T-2 toxin (T-2), fumonisins (FBs) including fumonisin B1 (FB1), zearalenone (ZEN) and ochratoxin A (Marin et al., 2013; Streit et al., 2012). Fifty years of research about these mycotoxins revealed their potential to cause acute and chronic toxic responses in humans and animals. Pigs are considered one of the most sensitive animal species due to their mainly cereal based diet and their specific characteristics related to the oral absorption and elimination of several mycotoxins. Many countries therefore regulated these highly prevalent mycotoxins in food and feed. Regarding animal feed, current EU legislation includes maximum levels for AFB1 (2002/32/EC), guidance values for DON, ZEN, OTA and FBs (2006/576/EC) and indicative values for T-2 and HT-2 toxin (2013/165/EU). This overview presents current findings on their global prevalence in feed, their exposure in pigs and related biomarkers for exposure, their immunomodulatory effects and consequences with respect to susceptibility to infectious diseases and vaccination efficiency.

Global mycotoxin contamination and occurrence

Contamination of food and feed commodities with mycotoxins is a worldwide problem. In 2019, a large dataset of mycotoxin concentrations in feed and a comprehensive assessment of regional trends of mycotoxin occurrence was published by Gruber-Dorninger et al. (2019). The global occurrence and co-occurrence of AFB1, FBs, ZEN, DON, OTA, and T-2 was assessed in 74,821 samples of finished feed and feed raw materials such as maize, wheat, barley, and soybean collected from 100 countries during a 10-year period. The authors compared mycotoxin occurrence in 15 geographic regions covering most of the globe and analyzed the year-to-year variation of mycotoxin concentrations in finished feed and maize from each region. Of the samples tested for ≥ 3 mycotoxins, 88% were contaminated with at least one mycotoxin. The *Fusarium* mycotoxins DON, FBs, and ZEN were most prevalent and were detected in 64%, 60%, and 45% of all samples, respectively. AFB1, T-2, and OTA were detected in 23%, 19%, and 15% of the samples, respectively. Fumonisins and DON showed the highest median concentrations, namely 723 µg/kg and 388 µg/kg, respectively. Most frequently observed mycotoxin mixtures were combinations of DON, ZEN, and FBs, as well as FBs and AFB1. In general, some mycotoxin combinations may show synergistic toxic effects, indicating that the simultaneous presence of mycotoxins may be more toxic than predicted from the mycotoxins alone (Alassane-Kpembi et al., 2017).

Table 1 shows the EU maximum levels or guidance values applicable for complementary and/or complete feedingstuffs for pigs (2002/32/EC, 2006/576/EC) and the global occurrence of AFB1, FBs, ZEN, DON, and OTA in 74,806 samples of finished feed and feed raw materials, shown for 14 geographical regions and as a percentage of samples exceeding the maximum levels or guidance values that are in place in the EU for pig feed (Gruber-Dorninger et al. (2019).

This global survey showed a high mycotoxin contamination prevalence, but with the majority of samples found to comply with the EU regulatory limits or guidance values for mycotoxins in pig feed. Therefore, acute mycotoxicoses resulting from the intake of high doses is rather rare in modern agricultural practice. Those acute toxic effects are characterized by well-described clinical signs, depending on the mycotoxin type, level and duration of exposure, animal species and age of the animal (Smith et al., 2005). As an example, exposure of pigs to high concentrations of DON causes abdominal distress, malaise, diarrhea, emesis and even shock or death. Exposure of pigs to FBs can lead to pulmonary edema due to cardiac insufficiency.

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Table 1. EU maximum levels or guidance values applicable for complementary and/or complete feedingstuffs for pigs (2002/32/EC, 2006/576/EC) and global occurrence of aflatoxin B1, fumonisins, zearalenone, deoxynivalenol, and ochratoxin A in 74,806 samples of finished feed and feed raw materials, shown for 14 geographical regions and as a percentage of samples exceeding these maximum levels or guidance values (from Gruber-Dorninger et al., 2019).

EU maximum levels or guidancevalues in pig feed	Aflatoxin B1 20 µg/kg	Fumonisins* 5,000 µg/kg	Zearalenone Piglets and gilts: 100 µg/kg Sows and fattening pigs:250 µg/kg	Deoxynivalenol 900 µg/kg	Ochratoxin A 50 µg/kg
Geographic region (n= total number of samples analysed)	Aflatoxin B1 % exceeding 20 µg/kg	Fumonisins* % exceeding 5,000 µg/kg	Zearalenone % exceeding 100 µg/kg	Deoxynivalenol % exceeding 900 µg/kg	Ochratoxin A % exceeding 50 µg/kg
Northern Europe (n=1,958)	0.4	0.0	6.2	21.5	0.2
Central Europe (n= 21,036)	1.0	1.3	13.0	20.4	0.3
Southern Europe (n=3,527)	2.1	3.3	11.8	11.7	0.9
Eastern Europe (n=2,382)	0.2	0.3	4.8	4.3	0.4
North America (n=5,471)	3.4	3.9	16.8	19.1	0.1
Central America (n=367)	0.0	3.8	10.7	8.1	0.0
South America (n=17,332)	1.3	8.4	13.1	5.1	0.8
Middle East/ North Africa (n=1,075)	3.5	1.1	8.6	5.6	0.9
Sub-Saharan Africa (n=208)	38.5	1.0	5.0	7.0	4.2
South Africa (n=1,077)	1.2	2.0	8.1	11.1	0.1
Oceania (n=1,695)	1.0	0.6	11.1	5.1	0.1
South Asia (n=1,136)	41.1	0.5	2.0	1.5	2.4
Southeast Asia (n=4,310)	20.9	2.0	10.1	4.8	0.4
East Asia (n=13,232)	6.6	3.9	27.3	20.6	0.3

*The EU guidance value in pig feed refers to the sum of fumonisins B1 and B2, but the prevalence data are shown for the sum of fumonisins B1, B2, and B3.

Global occurrence data show that livestock is continuously exposed to mycotoxins causing adverse health effects and reducing animal performance, and consequently causing an unavoidable economic impact. A meta-analysis of 85 papers published between 1968 and 2010, including 1,012 treatments and 13,196 pigs, associated mycotoxins with the performance and organ weights in growing pigs (Andretta et al., 2012). The magnitude of the effects varied with type and concentration of mycotoxin, gender, age and nutritional factors. The presence of mycotoxins was seen to reduce the feed intake by 18% and the weight gain in 21% compared with the control group. Deoxynivalenol and AF had the greatest impact on the feed intake and growth of pigs, reducing the feed intake by 26% and 16%, and the weight gain by 26% and 22%, respectively. The mycotoxin effect on growth proved to be greater in younger animals. In addition, the analysis showed that the greater part of the variation in weight gain was explained by the variation in feed intake (87%). The protein and methionine levels in diets could influence the feed intake and the weight gain in challenged animals. The weight gain in challenged pigs showed a positive correlation with the methionine level in diets (0.68). The mycotoxin effect on growth was greater in males compared with the effect on females as the reduction in weight gain was 15% in females and 19% in males. It was also reported that mycotoxins interfered with the relative weight of the liver, kidneys and heart (Andretta et al., 2012).

Considering the current contamination levels found in daily practice, it is clear that research should focus on the effects of low to moderate doses. Indeed, such realistic concentrations, even below regulatory limits can negatively affect animal performance (Kolawole et al., 2020).

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With respect to future trends and global warming, climate change is expected to have significant impacts on plant biogeography and fungal populations, with effects on mycotoxin patterns, as confirmed by predictive modeling approaches and field surveys. As an example, AFB1 is expected to increase in maize in Europe as a result of climate change, this prediction is based on modeling and is confirmed by field surveys (Battilani et al., 2013; Battilani et al., 2016; Leggieri et al., 2021).

The continuous development of more sensitive detection methods demonstrate that besides the regulated mycotoxins, so-called 'emerging mycotoxins' also frequently contaminate food and feed samples. These emerging mycotoxins are defined as mycotoxins which frequently contaminate food and feed, and could pose a health risk due to their toxic mechanisms and for which there is currently no legislation or guideline available. The use of multi-mycotoxin analytical methods demonstrates that the emerging *Fusarium* mycotoxins enniatins, beauvericin, moniliformin, and the *Alternaria* mycotoxins alternariol, alternariol monomethyl ether and tenuazonic acid are frequently detected in both food and feed (Streit et al., 2013).

Mode of action of major mycotoxins

Aflatoxins are mainly produced by *Aspergillus* species, such as *A. flavus*, *A. parasiticus* and *A. nominus* and are especially found in (sub)tropical regions. Aflatoxins and in particular AFB1 are notorious because they are considered the most potent naturally occurring carcinogens. AFB1 undergoes a hepatic epoxidation with the formation of aflatoxin B1-8,9-epoxide (AFBO). AFBO and the guanine part of DNA interact to form AFBO-guanine adducts, hence its hepatocellular carcinogenic properties (IARC class I carcinogen). Also malnutrition and growth impairmenthave been related to exposure to AFB1 (Rushing and Selim, 2018).

Fusarium fungi belong to one of the other most important phytopathogenic fungal genera. Major mycotoxins are trichothecenes A (for example T-2) and B (for example DON), FBs and ZEN. Trichothecenes share a common core characterised by the presence of an epoxide group, largely responsible for the mode of action and toxicity. DON inhibits protein synthesis by interfering with the termination step of the polypeptide chain. This is part of the elongation step of the protein synthesis and takes place in the 60S ribosomal unit. Acute intoxications with DON in pigs lead to abdominal pain, increased salivation, diarrhoea and emesis at doses of around 0.1-0.2 mg/kg BW in 10 kg weighing pigs (Forsyth et al., 1977). At lower doses a reduced feed intake and decreased weight gain are observedas well as growth retardation, immunotoxicity, impaired reproduction and development (Pestka, 2010). Both neuroendocrine factors and proinflammatory cytokines drive the anorexigenic effects of DON, as demonstrated by Girardet et al. (2011) who showed that DON can reach the brain after per os administration and act centrally on the anorexigenic/orexigenic balance.

T-2 is considered the most acute toxic substance of the trichothecenes and is known for its genotoxic and cytotoxic properties. It inhibits protein synthesis by interfering with the initiation of the polypeptide chain in the 60S ribosomal unit. Moreover, T-2 inhibits DNA and RNA synthesis and promotes the formation of reactive oxygen species. In swine, T-2 intoxication is established by alimentary toxic aleukia (ATA), necrotic-ulcerative inflammation of the digestive tract and some necrosis on the snout, lips and tongue. Also, growth retardation, reduced feed intake/refusal and decreased weight gain have been observed at low chronic dosage. T-2 exposure can also have reproductive and teratogenic effects in pigs (Adhikari et al., 2017).

FB1 is the most known representative of the FBs. It was discovered as the cause of pulmonary oedema and cardiac dysfunction in pigs in 1989 as thousands of pigs died in the USA related to contaminated corn (Osweiler et al., 1992). FB1 competitively inhibits ceramide synthase which results in the disruption of the ceramide biosynthesis and alteration of the sphingolipid metabolism, leading to accumulation of sphinganine (Sa) and sphingosine (So) in tissues, serum and urine (Pierron et al., 2016b). This impairs nutrient absorption, decreases daily weight gain and induces hepatotoxicity in pigs. Clinically the animals become anorectic with signs of encephalopathy (Haschek et al., 2001).

ZEN is a macrocyclic β-resorcylic acid lactone and is structurally similar to naturally-occurring oestrogens (Zinedine et al., 2007). Fertility and reproduction disorders are typically seen after administration of ZEN. Hyperestrogenism in pigs is already seen from doses as low as 0.06 and 0.15 mg/kg feed and manifests itself as reddening, hyperaemia and oedematous swelling of the vulva, enlargement of the uterus with cyst formation on ovaria and enlargement of the mammary glands (Zinedine et al., 2007). It can also cause vaginal or rectal prolapse. The symptoms are mostly seen in gilts since their concentrations of 17-β-oestradiol are lower compared to sows. In boars, atrophy of the testes, reduction of the concentration of spermatozoa and oedematous swelling of the preputium and mammarian complex are observed. Next to hyperestrogenism, also embryo toxicity and teratogenicity is observed in pigs after transfer of ZEN through the placenta.

OTA is produced by *Aspergillus* species, which primarily occur in (sub)tropical climate, but also *Penicillium* species

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present in colder climates can produce ochratoxins. The main producers are *A. ochraceus*, *A. niger* and *P. verrucosum*. The main toxicity of OTA is exerted by disturbing the protein synthesis. Therefore, the phenylalanine-like moiety of OTA acts as the amino acid itself and inhibits phenylalanine hydroxylase and phenylalanine tRNA transferase. This leads to nephrotoxic, hepatotoxic, teratogenic, neurotic, immunotoxic, genotoxic and cytotoxic effects (Malir et al., 2016; Pfohl-Leszkowicz and Manderville, 2007). In pigs, the disease is known as mycotoxic porcine nephropathy (MPN) and is associated with progressive interstitial fibrosis and regressive tubular changes with thickening of the basement membranes in kidneys. Also, a depression and reduction in feed intake has been observed (Battacone et al., 2010).

Mycotoxin exposure and biomarkers for exposure in pigs

The susceptibility of an animal species towards the effects of mycotoxins in part depends on the absorption, distribution, metabolism and excretion (ADME processes) of the toxins. For example, pigs rapidly convert ZEN primarily to α-zearalenol (α-ZEL), while chickens to β-zearalenol (β-ZEL) (Malekinejad et al., 2006). As α-ZEL has higher binding affinity towards estrogen receptors than ZEN and β-ZEL, this explains the high sensitivity of pigs to ZEN. For DON, a similar difference in detoxification capability can explain why ruminants are more resistant to its effects. DON is rapidly converted to its de-epoxy derivate in the rumen of healthy cattle (Pestka, 2007). However in pigs, although de-epoxidation occurs in the distal intestine, DON is rapidly and almost completely absorbed from the first part of the intestine, hence de-epoxidation is not a significant detoxification pathway in pigs, most likely explaining their higher sensitivity towards DON (Eriksen et al., 2002).

Mycotoxin exposure in pigs is mainly investigated by feed analysis. However, it is well known that so called 'hot spots' are responsible for an uneven distribution and non-proportional spread of mycotoxins in feed, hampering representative sample collection and evaluation of mycotoxin exposure in animals. On the other hand, modified or conjugated forms of mycotoxins also present in feed are normally not included in routine feed analysis, however, they are converted back to their free forms during digestion and in consequence they contribute to the adverse effects related to mycotoxin exposure. For instance, deoxynivalenol-3-glucoside, which can be as common as DON in feed, is converted to free DON in pigs (Broekaert et al., 2017). This *in vivo* conversion has also been described for zearalenone-14-glucoside in pigs (Catteuw et al., 2019).

These hampering factors related to feed analysis can be overcome by the analysis of biological matrices such as plasma, urine and/or faeces, since exposure can be assessed on an individual level and clinical signs can be directly related to exposure. In human mycotoxicology, biomarker-driven research has been proven a successful method for the assessment of mycotoxin exposure by biomonitoring parent toxins and/or metabolites in biological matrices like urine and blood (Vidal et al., 2018). For blood collection, innovative microsampling strategies are of particular interest, since they require a smaller sample volume (≤ 50 μL) and are often less painful and stressful, hence their application in the paediatric population too. Furthermore, they have generally fewer requirements on handling and storage. Currently, dried blood spot (DBS) sampling is the most frequently used dried microsampling technique, while the volumetric absorptive microsampling (VAMS) technique was recently introduced to reduce volume variations seen with DBS, owing to alteration in haematocrit values.

We recently assessed the potential of DBS sampling in mycotoxin monitoring in pigs, at the farm level. An ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method targeting 23 mycotoxin biomarkers via DBS was validated for pigs (Lauwers et al., 2019), and was further extended to 36 biomarkers. These biomarkers comprised the parent mycotoxins, as well as relevant phase I and II metabolites. In total 489 DBS samples from swine (piglets, sows and fattening pigs) originating from 20 different countries around the globe were analysed, as well as the feed consumed at the moment of blood collection. Results demonstrated that 97% of the studied animals were co-exposed to two or more mycotoxins, and more than 50% of which were exposed to six or more mycotoxins (Vidal et al., in preparation). Interestingly, emerging mycotoxins produced by *Alternaria* and *Fusarium* spp. in combination with DON and OTA were the most predominant within the 31 mycotoxin biomarkers identified (ranging from trace level up to 2,354 ng/mL). Namely, tenuazonic acid was the most prevalent mycotoxin in blood (68% in swine farms), followed by OTA (57%) and enniatin B1 (57%). Some tendencies between mycotoxin exposure and reproductive health status were identified. For example, exposure to estrogenic mycotoxins like ZEN and the *Alternaria* mycotoxin alternariol was detected in 79% and 81% of sows presenting agalactia and swollen vulva, respectively.

Using the biomarkers analysis in plasma of sows and their piglets, Van Limbergen et al. (2017) showed a possible involvement of DON in the development of neonatal tail necrosis in piglets. Ten farms with and 10 farms without symptoms of neonatal tail necrosis were selected. On each farm, samples of water, feed and blood were collected from 5 sows and 2 piglets per sow. The concentration of DON in sow feed was significantly higher in case- than in control herds: 484 *vs* 257 μg/kg feed. The same trend was also observed in sow plasma. Moreover, positive correlations

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between DON concentrations in feed and sow plasma (r=0.52) or piglet plasma (r=0.33) were found, indicating a possible involvement of DON in the pathogenesis of tail necrosis.

Another interesting application of the biomarkers analysis in pigs, is in the assessment of the *in vivo* efficacy of candidate mycotoxin detoxifiers, as demonstrated by Devreese et al. (2014). The use of suitable biomarkers in such efficacy studies is however currently limited, as mostly the efficacy is either determined by *in vitro* studies or by measuring nonspecific parameters such as performance.

Immunomodulatory properties of mycotoxins in pigs

The main targets of mycotoxins are rapidly proliferating and differentiating cells and tissues, with a high protein turnover, including the small intestine, liver and immune cells. The capacity of mycotoxins to alter normal gut and immune function has been of particular interest, especially because this can be seen at contamination below levels that induce a negative impact on performance (Grenier and Applegate, 2013; Pinton and Oswald, 2014).

The intestinal mucosa acts as a first barrier after oral intake of mycotoxins, preventing the entry of foreign antigens including food proteins, xenobiotics (such as drugs and toxins), commensal microbiota and pathogens into the underlying tissues (Oswald, 2006). The mucosal immunity, which consists of an innate and adaptive immune system, can be affected by mycotoxins (Figure 1) (Antonissen et al., 2014; Bouhet and Oswald, 2005).
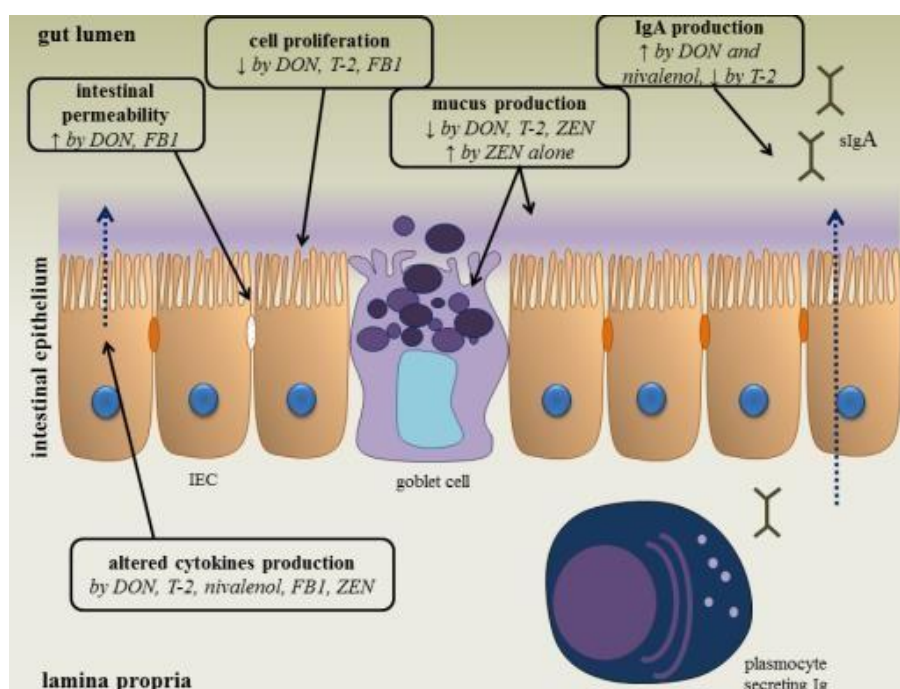
Figure 1. The effect of *Fusarium* mycotoxins on the intestinal epithelium. A variety of *Fusarium* mycotoxins alter the different intestinal defense mechanisms including epithelial integrity, cell proliferation, mucus layer, immunoglobulins (Ig) and cytokine production. (IEC: intestinal epithelial cell) (from Antonissen et al., 2014, based on Bouhet and Oswald, 2005).

An important component of the innate immune system are the intestinal epithelial cells, which are interconnected by tight junctions, and covered with mucus, produced by goblet cells. By measuring the transepithelial electrical resistance (TEER), several *in vitro* and *ex vivo* studies indicate that DON and FB1 are able to increase the permeability of the intestinal epithelial layer of human, porcine and avian origin (Bouhet et al., 2004; Pinton et al., 2009). Also the viability and proliferation of animal and human intestinal epithelial cells can be negatively affected by several mycotoxins (Bouhet and Oswald, 2005; Sergent et al., 2006). Their effect on mucus production is variable: co-exposure of low doses of DON, T-2 and ZEN reduces the number of goblet cells in pigs (Obremski et al., 2008), but ZEN given alone at higher doses increases the activity of goblet cells (Obremski et al., 2004). Several mycotoxins are also able to modulate the production of cytokines *in vitro* and *in vivo* (Bondy and Pestka, 2000; Bouhet and Oswald, 2005), like FBs that decrease the expression of IL-8 in an intestinal porcine epithelial cell line (IPEC-1). The specific effects of DON and other type B trichothecenes on the intestinal immune response have been reviewed by Pinton and Oswald (2014). In porcine jejunal explants, DON mainly drives the intestinal immune system towards a Th17 response elicited by the Th17 helper lymphocytes, which are mediators of the mucosal immunity, the defense against extracellular pathogens and autoimmunity. Besides these direct effects, DON also potentiates the effects of other

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proinflammatory stimuli, such as TLR-4 ligands, lipopolysaccharide (LPS) and bacteria on immune cells (Mbandi and Pestka, 2006; Zhou et al., 1999). DON modulates the production of several proinflammatory cytokines following a 48-h treatment of IPEC-J2 cells (Wan et al., 2013). The consequence of such production is the modulation of the intestinal tight junction barrier, potentially leading to an increased translocation of luminal antigens (Al-Sadi et al., 2009). The induction of proinflammatory cytokines, such as IL-6 by macrophages, is directly linked to the differentiation of B-cells and to the stimulation of IgA secretion (Pestka, 2003). *In vivo* and *in vitro*, DON upregulates the IL-6 expression that drives the differentiation of IgA-committed B-cells to IgA secretion (Pinton et al., 2008).

In conclusion, several mycotoxins, and especially trichothecenes, induce intestinal pathologies in pigs, including necrosis of the intestinal epithelium. They may also disturb the barrier function, potentially leading to the increased translocation of pathogens and an increased susceptibility to enteric infectious diseases. DON modulates the immune responsiveness of the intestinal mucosa, interacts in the cross-talk between epithelial cells and intestinal immune cells and hence may represent a predisposing factor to inflammatory diseases (Maresca and Fantini, 2010).

After oral intake, several mycotoxins can cross the intestinal epithelium and reach the systemic compartment, affecting the global immune system as well. Exposure can either result in immunostimulatory or immunosuppressive effects depending on the age of the host and exposure dose and duration (Corrier, 1991). Mycotoxin-induced immunomodulation may affect innate and adaptive immunity by an impaired function of macrophages and neutrophils, a decreased T- and B-lymphocyte activity and antibody production (Bondy and Pestka, 2000; Oswald et al., 2005). The systemic immunomodulatory effects of major mycotoxins in pigs were reviewed by Pierron et al. (2016a), as summarized below.

AF impair both the innate and acquired immune responses (Meissonnier et al., 2006, Weaver et al., 2013). AFB1 impairs cell-mediated immunity by a dysregulation of the antigen-presenting capacity of dentritic cells (Mehrzad et al., 2014; Mehrzad et al., 2015). An alteration of the inflammatory response has also been reported in pigs exposed to AF (Chaytor et al., 2011). A reduced synthesis of proinflammatory cytokines and an increase of anti-inflammatory cytokines was demonstrated in weaned piglets fed for 4 weeks with low doses of AF (Marin et al., 2002). *In utero* exposure of piglets to AF altered the functional capacities of both macrophages and neutrophils (Silvotti et al., 1997).

Type B trichothecenes, including DON, have the capacity to up- and down-regulate immune functions by disrupting intracellular signaling within leukocytes (Pestka, 2010). Depending on the dose, frequency and duration of exposure, DON will have either an immunostimulatory or immunosuppressing effect (Pestka et al., 2004). DON is able to induce an inflammatory response by acting on the ribosome, inducing a ribotoxic stress response which activates the MAPK pathway, eliciting expression of inflammation-related genes as proinflammatory cytokines (Pestka et al., 2004; Pestka, 2010). More recently, Liu et al. (2020) also studied the bidirectional immune effects of DON due to different exposure doses, both in weaned piglets and porcine alveolar macrophages (PAM). The results revealed that low doses of DON increase the expression of TNF-α and IL-6 in piglets and PAM, promote the chemotaxis and phagocytosis of PAM and transform macrophages to M1 phenotype. Conversely, high doses of DON increase the expression of TGF-β and IL-10 in piglets and PAM, inhibit the chemotaxis and phagocytosis of PAM and induce macrophages M2-type polarization. Mechanistically, DON exposure significantly activates the TLR4/NFκB pathway at low doses and induces mitophagy-mediated mitochondrial dysfunction at high doses, both seen *in vitro* and *in vivo*. Type A trichothecenes such as T-2 are cytotoxic molecules and potent protein inhibitors.

In *in vitro* and *in vivo* experiments, FB1 modifies the Th1/Th2 (T-helper 1/T-helper 2) cytokine balance in pigs similar to an impaired humoral response (Marin et al., 2006, Taranu et al., 2005). Studies have also demonstrated that FB1 influences the inflammatory response. For example, incubation of PAM with FB1 led to a significant reduction of the number of viable cells and cell death by apoptosis (Liu et al., 2002). An *in vivo* experiment on pigs exposed to FBs (6 mg/kg feed for 5 weeks) showed a decrease of IL-1β and IL-6 genes expression in spleen tissue (Grenier et al., 2011). FB1 also impairs on the maturation of antigen presenting cells *in vivo* by reducing the intestinal expression of IL-12p40 and decreasing the upregulation of major histocompatibility complex class II molecule (MHC-II) with a reduction of T cell stimulatory capacity upon stimulation (Devriendt et al., 2009).

Gilts fed OTA-contaminated feed had a reduced cutaneous basophil hypersensitivity response to phytohemagglutinin, reduced delayed hypersensitivity to tuberculin, decreased stimulation index for lymphoblastogenesis, decreased IL-2 production when lymphocytes were stimulated with concanavalin A, and a decreased number and phagocytic activity of macrophages. OTA was shown to be toxic on purified lymphocytes of pigs with a half maximal inhibitory concentration (IC50, concentration producing 50% inhibition of cell proliferation), of 1.3 μM (Keblys et al., 2004). OTA also showed an impact on the cytokine expression. An experiment on weaned pigs that ingested an OTA contaminated diet (181 ng/g feed) showed an increased level of TNF-α and IL-10 in plasma, with a decreased capacity to respond with cytokine expression to an *ex vivo* challenge to LPS (Bernardini et al., 2014). By contrast, OTA has no effect on total and specific immunoglobulin concentrations (Harvey et al., 1992).

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Only few papers described the effect of ZEN on immunity (Eriksen and Alexander, 1998). In pigs, exposure of intestinal epithelial cells to ZEN (25 μM) has a tendency to increase the synthesis of inflammatory cytokines IL-8 and IL-10 (Marin et al., 2015). Sows exposed to high concentration of ZEN (5–250 mg/kg feed or 200–1000 μg/kg BW per day) can develop a chronic inflammation of the genital tract (EFSA, 2011).

Mycotoxins and susceptibility to infectious diseases in pigs

The immune system is primarily responsible for defense against invading organisms. Many papers clearly indicate a negative influence of several mycotoxins on the intestinal function and immune system. Since the intestinal tract is also a major portal of entry to many enteric pathogens and their toxins, mycotoxin exposure could increase the animal susceptibility to these pathogens. Furthermore, as a consequence of the systemic immunomodulatory properties of many mycotoxins, an increased susceptibility to infectious diseases and a decreased vaccine efficacy may be seen.

Antonissen et al. (2014) summarized the *in vitro* (Table 2) and *in vivo* interaction between major ~~mycotoxin~~s and infectious diseases in animals, and Pierron et al. (2016a) reviewed the *in vivo* data about the impact of major mycotoxins on the susceptibility to infectious diseases in pigs (Table 3).

Table 2. Interaction between major mycotoxins and infectious diseases in pigs: *in vitro* studies (adapted from Antonissen et al., 2014).

Myco toxin	Exposure dose	Exposure period	Cell line	Pathogen	Effect	Reference
DON or T-2	> 25 ng DON/mLor 5 ng T-2/mL ≥ 0.75 µg DON/mL or ≥ 2.5ng T-2/mL	24 h	undifferentiated IPEC-J2 differentiated IPEC-J2	*Salmonella* Typhimurium	↑ invasion	Vandenbroucke et al., 2011; Verbrugghe et al., 2012
DON or T-2	0.5 µg DON/mL or ≥ 1.0 ng T-2/mL	24 h	differentiated IPEC-J2	*Salmonella* Typhimurium	↑ translocation	Vandenbroucke et al., 2011; Verbrugghe et al., 2012
DON or T-2	0.025 µg DON/mL or 1 ng T-2/mL	24 h	PAM	*Salmonella* Typhimurium	↑ invasion	Vandenbroucke et al., 2009; Verbrugghe et al., 2012
DON	1.5 - 15 µg/mL	48 h	IPEC-J1	*Escherichia coli* (SEPEC)	↑ translocation	Pinton et al., 2009
ZEN	25 µM	1 h or 24h	undifferentiated IPEC-1	*Escherichia coli* (ETEC)	↑ expression TNF-α, IL-1β, IL-6, IL-8, IL-10, IFN-γ	Taranu et al., 2015; Braicu et al., 2016

DON=deoxynivalenol ; T-2=T-2 toxin; IPEC=intestinal porcine epithelial cell, PAM=porcine alveolar macrophage, SEPEC=septicemic *Escherichia coli*, ETEC=enterotoxigenic *Escherichia coli*

Table 3. Influence of major mycotoxins on the susceptibility to infectious diseases in pigs: *in vivo* studies (adapted from Antonissen et al., 2014; Pierron et al., 2016a).

Myco toxin	Exposure dose	Exposure period	Age	Pathogen	Effect compared to negative control	Reference
AFB1	0.07 and 0.14 mg/kg	32 d		*Brachyspira hyodysenteriae*	↓ incubation period for dysentery, ↑ diarrhea and dysentery time, ↑ death, visible clinical signs and lesions of dysentery at necropsy	Joens et al., 1981
AF	1.3 mg/kg feed	25 d		*Erysipelothrix rhusiopathiae*	↑ severity of bacterial infection	Cysewski et al., 1978
DON	2.5 mg/kg feed	3 w	3 w	PCV2	↑ viremia and lung viral load no clinical effect	Savard et al., 2015a

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DON	3.5 mg/kg feed	3 w	5 w	PRRSV	↓ weight gain, ↑ lung lesions and mortality, no effect on viral replication	Savard et al., 2014
DON	1 µg/mL	6 h	5 w	*Salmonella* Typhimurium	synergistic ↑ gene expression IL-12, TNF-α, IL-1β, IL-8, MCP-1 and IL-6	Vanden broucke et al., 2011
DON	1.5 mg/kg feed	35 d	5 w	*Mycoplasma hyopneumoniae*	no increase in severity of infection	Michiels et al., 2018
T-2	15 and 83 µg/kg feed	23 d	3 w	*Salmonella* Typhimurium	↓ colonization of the cecum	Verbrugghe et al., 2012
FB1 and FB2	8.6 mg FB1 and 3.2 mg FB2/kg feed	9 w	4 w	*Salmonella* Typhimurium	modification of microbiota profiles	Burel et al., 2013
FB1	0.5 mg/kg BW	6 d	3 w	*Escherichia coli* (SEPEC)	↑ intestinal colonization; ↑ translocation to the mesenteric lymph node, lung, liver and spleen	Oswald et al., 2003
FB1	1 mg/kg BW	10 d	3-4 w	*Escherichia coli* (ETEC)	intestinal infection prolonged; impaired function of intestinal antigen presenting cells	Devriendt et al., 2009
FB1, FB2 and FB3	20 mg FB1, 3.5 mg FB2 and 1.9 mg/kg feed	42 d	3 d	*Mycoplasma hyopneumoniae*	↑ severity of the pathological changes	Pósa et al., 2013
FB1	10 mg/kg feed	24 d	3 d	*Bordetella bronchiseptica* and *Pasteurella multocida* (type D)	↑ extent and severity of the pathological changes	Pósa et al., 2011
FB1	0.5 mg/kg BW	7 d	pig let	*Pasteurella multocida* (type A)	↑ total number of cells, number of macrophages and lymphocytes in BALF ↑ gross pathological lesions and histopathological lesion of lungs	Halloy et al., 2005
FB1	12 mg/kg BW	18 d	1 m	PRRSV	↑ histopathological lesions of lungs	Ramos et al., 2010
FB1	20 mg/kg feed	26 d	2 w	*Pasteurella multocida* (type A)	↑ lung pathology	Kovács et al., 2016
OTA	3 mg/kg feed	3 w	6 w	*Brachyspira hyodysenteriae* and *Campylobacter coli*	spontaneous Salmonellosis, clinical and patho-morphological changes, change of hematological and biological parameters	Stoev et al., 2000
OTA	75 µg/kg feed	42 d	6 w	PCV2	↑ PCV2 replication in serum and tissues	Gan et al., 2015

AF=aflatoxins; AFB1=aflatoxin B1; DON=deoxynivalenol; FB1=fumonisin B1; FB2=fumonisin B2; FB3=fumonisin B3; OTA=ochratoxin A; BW=body weight; SEPEC=septicemic *Escherichia coli*; ETEC=enterotoxigenic *Escherichia coli*; PRRSV= Porcine Reproductive and Respiratory Syndrome Virus; PCV2=porcine circovirus type2.

Table 3 shows a negative impact of several highly prevalent mycotoxins on the susceptibility of major infectious diseases in pigs, hence they can be considered as predisposing factors in the development of the diseases. Although, it must be mentioned that in the majority of the studies, the contamination levels tested were exceeding current EU maximum levels or guidance values in complete and complementary feedstuffs for pigs. The exact outcome of co-exposure to mycotoxins and swine pathogens is difficult to predict. Published data show an influence of mycotoxin exposure on the bacterium, the host cells and the host–pathogen interaction. Depending on the characteristics of the mycotoxin exposure, one of these effects will determine the final outcome of the interaction between mycotoxins and pathogens.

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Considering Salmonellosis, the impact has been mechanistically proven at several levels in the pathogenesis. An infection with *Salmonella* generally occurs in three stages: adhesion to the intestinal wall, invasion of the gut wall and dissemination to mesenteric lymph nodes and other organs. Feeding pigs a *Fusarium* mycotoxin contaminated diet influences the intestinal phase of the pathogenesis of *Salmonella* Typhimurium infections, as illustrated in Figure 2. Non-cytotoxic concentrations of DON and T-2 enhance intestinal *Salmonella* invasion and increase the passage of *Salmonella* Typhimurium across the epithelium (Vandenbroucke et al., 2009; Verbrugghe et al., 2012). On the other hand, chronic exposure of specific pathogen free pigs to naturally FBs contaminated feed had no impact on *Salmonella* Typhimurium translocation (Burel et al., 2013), demonstrating specific mycotoxin-dependent mechanisms. Once *Salmonella* has invaded the intestinal epithelium, the innate immune system is triggered and the porcine gut starts to produce several cytokines. Both DON and T-2 and *Salmonella* affect the innate immune system. Vandenbroucke et al. (2011) showed that low concentrations of DON could potentiate the early intestinal immune response induced by *Salmonella* Typhimurium infection. Co-exposure of the intestine to DON and *Salmonella* Typhimurium resulted in increased expression of several cytokines, for example responsible for the stimulation of the inflammatory response (TNF-α) and T-lymphocyte stimulation (IL-12). The authors suggested that the enhanced intestinal inflammation could be due to a DON induced stimulation of *Salmonella* Typhimurium invasion in and translocation across the intestinal epithelium (Vandenbroucke et al., 2011).

Fusarium mycotoxins also affect the systemic part of the *Salmonella* Typhimurium infection in pigs. After the intestinal phase of the pathogenesis, *Salmonella* can spread to the bloodstream using the host macrophage to establish the systemic infection. After bacterial uptake by the macrophage, *Salmonella* can survive and even proliferate in this cell. Exposure of macrophages to non-cytotoxic concentrations of DON and T-2 promotes the uptake of *Salmonella* Typhimurium (Figure 3). *Salmonella* entry in host cells involves a complex series of actin cytoskeletal changes. Macrophage invasion coincides with membrane ruffling, followed by bacterium uptake and formation of a *Salmonella* containing vacuole. Vandenbroucke et al. (2009) showed *in vitro* that DON enhances *Salmonella* Typhimurium engulfment, since low concentrations of DON modulate the cytoskeleton of macrophages through ERK1/2 F-actin reorganization resulting in an enhanced uptake of *Salmonella* Typhimurium in PAM (Figure 3). Non-cytotoxic concentrations of the *Fusarium* mycotoxins DON and T-2 did not affect the intracellular proliferation of *Salmonella* Typhimurium in porcine macrophages (Figure 2) (Vandenbroucke et al., 2009; Verbrugghe et al., 2012).

In addition to the effects of *Fusarium* mycotoxins on the host susceptibility to a *Salmonella* Typhimurium infection, these mycotoxins also modulate the bacterial metabolism. Although no effect of DON or T-2 on the growth of *Salmonella* Typhimurium is detected, DON and T-2 modulate the *Salmonella* gene expression (Vandenbroucke et al., 2009; Verbrugghe et al., 2012). The enhanced inflammatory effect following exposure to DON is more likely a result of the toxic effect of the mycotoxin on the intestine, than on the bacterium. Only high concentrations of DON increase the bacterial expression of regulators of *Salmonella* pathogenicity island (SPI)-1 and SPI-2, respectively *hilA* and *ssrA*. SPI-1 consists of genes coding for bacterial secretion systems necessary for invasion, while SPI-2 genes encode essential intracellular replication mechanisms. For T-2 the toxic effects on the bacterium itself are probably more pronounced than the host cell-mediated effects resulting in a reduced *in vivo* colonization in pigs. Low concentrations of T-2 cause a reduced motility of *Salmonella* and a general down regulation of genes involved in *Salmonella* metabolism, genes encoding for ribosomal proteins and SPI-1 genes (Verbrugghe et al., 2012).
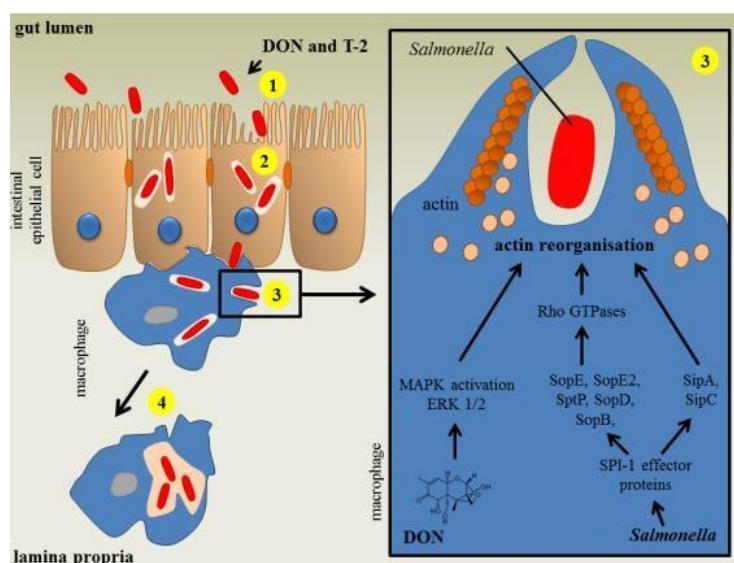

Figure 2. The impact of deoxynivalenol and T-2 toxin on a *Salmonella* Typhimurium infection in pigs. *In vitro*, deoxynivalenol (DON) and T-2 toxin (T-2) promote *Salmonella* invasion (1) and transepithelial passage (2) of an IPEC-J2 cell layer. Subsequently, the bacterium can spread to the bloodstream using the host macrophage to

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establish the systemic infection. *In vitro*, DON and T-2 enhance *Salmonella* uptake (3) in porcine alveolar macrophages. The *Salmonella* invasion of macrophages coincides with membrane ruffling, caused by actin cytoskeletal changes. Activation of host Rho GTPases by the *Salmonella* pathogenicity island (SPI)-1 type 3 secretion system effector proteins SopB, SopE, SopE2 and SopD leads to actin cytoskeleton reorganization. After *Salmonella* internalization has occurred, the bacterium injects the effector protein SptP which promotes the inactivation of Rho GTPases. The bacterium can also modulate the actin dynamics of the host cell in a direct manner through the bacterial effector proteins SipA and SipC. The mycotoxin DON enhances the uptake of *Salmonella* in macrophages through activationof the mitogen activated protein kinase (MAPK) ERK1/2 pathway, which induces actin reorganizations and membraneruffles. DON and T-2 do not affect intracellular bacterial proliferation (4) (from Antonissen et al., 2014).
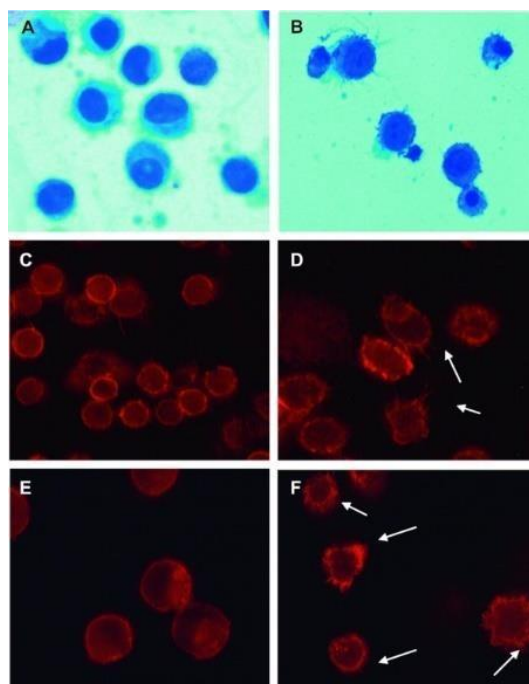
Figure 3. Haemacolor staining of porcine alveolar macrophages (PAM) whether or not exposed to 0.025 µg/mL of DON. Figure A shows PAM not exposed to DON, whereas Figure B shows DON exposed macrophages with marked membrane ruffling. Figures C–F show fluorescence microscopic pictures of actin filament arrangement in PAM either treated with cell medium, 0.025 µg/mL of DON, 10 µM of the ERK1/2 inhibitor U0126 ethanolate in combination with 0.025 µg/mL of DON or with 50 ng/mL of the ERK1/2 activator PMA, for 24 h. Control macrophages (C) and macrophages exposed to U0126 ethanolate in combination with DON (E) demonstrated a normal distribution of F-actin. DON (D) and PMA (F) exposed macrophages demonstrated increased formation of membrane ruffles, indicated by white arrows (from Vandenbroucke et al., 2009).

Mycotoxins and vaccination efficiency in pigs

As a consequence of the systemic immunomodulatory properties of many mycotoxins, a decreased vaccine efficacy may be seen, as summarized in Table 4 (Pierron et al., 2016a).

Experimentally, in a pig model vaccinated with the model antigen ovalbumin (OVA), AFB1 exposure had no major effect on the humoral immunity with unchanged plasma concentrations of total immunoglobulin A (IgA), IgG and IgM and the specific anti-OVA IgG. In these animals, the toxin exposure did not impair the mitogenic response of lymphocytes but delayed and decreased the OVA-specific proliferation, suggesting an impaired lymphocyte activation in pigs exposed to AFB1 (Meissonnier et al., 2008b). Similarly, in pigs vaccinated with *Mycoplasma*, the exposure to lower levels of AFB1 did not modulate the antigen-specific and total antibody response (Marin et al., 2002).

As mentioned above, in pigs, an increase of IgA in the serum of animals receiving DON contaminated feed has been observed (Pinton et al., 2008). In animals immunized with OVA, the specific immune response was investigated during a DON exposure inducing no feed refusal or reduced body weight gain. Ingestion of DON increased the plasma concentration of total and anti-OVA IgA titers. DON did not modulate lymphocytes proliferation after mitogenic stimulation, but the toxin had a biphasic effect on the OVA-specific lymphocyte proliferation: an up-regulation in the days after OVA immunization but a down-regulation in the weeks following (Pinton et al., 2008).

Another study on pigs immunized with OVA showed an increase of anti-OVA IgG titers, after 42 days of exposure to a DON contaminated diet. Simultaneously, the expressions of chemokines involved in inflammatory reactions (IL-8,

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chemokine (C-X-C motif) ligand 20 (CXCL20), IFN-γ) were up-regulated. DON also up-regulated the gene of major antioxidant glutathione peroxidase 2 (GPX-2) and down-regulated expression of genes encoding enzymatic antioxidants including GPX-3, GPX-4 and superoxide dismutase 3 (SOD-3), involved in oxidative stress (Lessard et al., 2015).

Type A trichothecenes such as T-2 are cytotoxic molecules and potent protein inhibitors. In pigs immunized with OVA, subclinical doses of T-2 induced an early and transient increase of total IgA plasma concentration but a decrease in the anti-OVA IgG titer (Meissonnier et al., 2008a). For higher doses of exposure, T-2 had been previously shown to decrease both the mitogenic and the antigen-specific lymphocytes proliferation following a horse globulin immunization (Rafai et al., 1995).

With pigs vaccinated against *Mycoplasma* and exposed to FB1 (8 mg/kg feed for 4 weeks), a sex-related difference in the specific immune response has also been observed. In male pigs but not for female ones, exposure to the toxin reduced the vaccine-specific antibody titer (Marin et al., 2006). However, ingestion of contaminated feed had no effect on the serum concentrations of total IgG, IgA, and IgM.

Table 4. Influence of major mycotoxins on the vaccination efficacy in pigs (adapted from Pierron et al., 2016a).

Myco toxin	Exposure dose	Antigen	Effect compared to negative control	Reference
AF	1.3 mg/kg feed	*Erysipelothrix rhusiopathiae*	impaired development of acquired immunity	Cysewski et al., 1978
AFB1	385-1,807 µg/kg feed	OVA	decreased and delayed cell-mediated immunity	Meissonnier et al., 2008b
DON	3.5 mg/kg feed	OVA	increased OVA-primary IgG antibody response	Lessard et al., 2015
DON	2.5-3.5 mg/kg BW	PRRSV	decreased PRRSV post-vaccinal viremia and reduced vaccinal efficacy	Savard et al., 2015b
DON	1.5-2.4 mg/kg feed	PRRSV and PCV2	decreased anti-PRRSV IgG response	Lo Verso et al., 2021
DON	2.2-2.5 mg/kg feed	OVA	increased concentration of OVA specific IgA and IgG	Pinton et al., 2008
DON	0.6-4.7 mg/kg	human serum albumin, sheep red blood cells, paratuberculosis vaccine, tetanus toxoid and diphtheria toxoid	dose-dependent reduction in secondary antibody response to tetanus toxoid	Overnes et al., 1997
DON + ZEN	2.1-3.2 mg DON/kg feed and 0.06-0.25 mg ZEN/kg feed	parvovirus	no effect	Gutzwiller et al., 2007
DON or FB1	3 mg DON/kg feed or 6 mg FB1/kg feed	OVA	reduced anti-OVA antibody production with a decrease of lymphocytes proliferation	Grenier et al., 2011
T-2 toxin	1,324-2,102 µg/kg feed	OVA	reduced anti-OVA antibody production without significant alteration to specific lymphocyte proliferation	Meissonnier et al., 2008a
FB1	8 mg/kg BW	*Mycoplasma agalactiae*	decreased specific antibody titer	Taranu et al., 2005
OTA	1 mg/kg feed	*Salmonella choleraesuis*	immunosuppression and delayed response to immunization	Stoev et al., 2000
OTA or FB1	0.5 mg OTA/kg feed or 10 mg FB1/kg feed	Suid herpesvirus 1 (Aujeszky disease)	decreased anti-SuHV1 antibody production after vaccination	Stoev et al., 2012

AF=aflatoxins; AFB1=aflatoxin B1; DON=deoxynivalenol; ZEN=zearalenone; FB1=fumonisin B1; OTA=ochratoxin A; BW=body weight; OVA=ovalbumin; PRRSV=porcine reproductive and respiratory syndrome virus; PCV2=porcine circovirus type2

Conclusions

Global mycotoxin monitoring in feed shows the occurrence of AFB1, FBs, ZEN, DON, OTA, and T-2, with 88% of samples being co-contaminated with at least one mycotoxin. This demonstrates the co-occurrence to multiple mycotoxins is daily practice, and is a major threat to pig health, welfare and performance. At the individual level in pigs, this co-occurrence is also confirmed by analysis of suitable biomarkers for exposure in blood. In general, the intestine is the first barrier that can be exposed to mycotoxins upon ingestion of contaminated feed. Several mycotoxins alter the intestinal barrier, impair the immune response, reduce feed intake and weight gain. Their presence in feed increases the translocation of bacteria; and the immunomodulatory properties of mycotoxins can also enhance the susceptibility to infectious diseases and reduce the vaccination efficacy.

Analysis of feed demonstrates that besides the six regulated mycotoxins mentioned, so-called emerging mycotoxins like the *Fusarium*-derived enniatins (mainly enniatin B1) and the *Alternaria* mycotoxin tenuazonic acid are frequently detected as well, also in the blood of pigs. There is currently no legislation or guideline available for these emerging mycotoxins in feed. Moreover, regulations that are in place for feed only apply to single mycotoxins, not to their co-occurrence.

Hence, current and future challenges in mycotoxin-related research are the inclusion of real-life exposure scenarios taking into account realistic concentrations and co-contamination. This might steer the further elaboration of regulatory levels in feed, and the development and commercialization of effective mycotoxin detoxifying agents to boost pig health and performance and to improve the sustainable use of crops.

Ultimately, an animal exposome network could be developed, taking not only fungal infections and intoxications into account, but also bacterial (and their endotoxins), viral, protozoal, and parasitic infections as well as environmental stressors.

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Update on ASF diagnosis and current circulating strains

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Summary

Knowledge of and attempts to control African swine fever (ASF) date back at least a century and it is now the first disease for which a viral etiology, ASF virus (ASFV), threatens the global swine industry in all over the world and affects the five continents. ASFV infects domestic and wild members of the Suidae family, causing a wide variety of symptoms, from chronic or persistent infection to acute haemorrhagic fever, causing up to 100% mortality in their sharper forms. There is no commercialized vaccine available against the ASFV, and current control measures consist of strict animal quarantine and culling procedures. The virus is very stable and spreads easily through infected pigs, contaminated pork products, and fomites, or by transmission by the *Ornithodoros* vector. The establishment of endemic ASFV infections in wild boar populations further complicates the control of the disease. Since its first description in Kenya in 1921, ASFV remained exclusively on the African continent until the end of the 1950s. It was in 1957 that ASFV emerged for the first time in Europe, spreading to South America, but was eradicated in mostly in the mid-1990s. In 2007, a highly virulent genotype II ASFV strain emerged in the Caucasus region and subsequently spread to the Russian Federation and Europe, where it has continued to circulate and spread. In 2018, ASFV jumped to China and spread to several neighbouring countries in Southeast Asia. More recently, and after 40 years of silence, the ASFV emerged in America, affecting the Dominican Republic and Haiti in 2021. The high morbidity and mortality associated with ASFV, the lack of an effective vaccine, and the complexity of the virus, as well as its epidemiology, make this pathogen a serious threat to the global swine industry and national economies. Topics covered by this review include the genetic characteristics of ASFV, its biological properties, with particular attention to the evolution of virulent to moderate and attenuated, strains, current and future diagnostic strategies, diagnostic gaps and their relevance.

ASFV genetics, epidemiology and genotype II epidemic history

The ASFV genome is approximately 170 to 190 kilobase (kb) pair and is divided into the left variable region (38 to 48 kb), the conserved central region (approximately 125 kb), and the right variable region (13 to 22 kb). The differences in size between the strains are due to insertions or deletions of the genes of the 5 multigene families (MGF), although variations in the conserved central region related to single nucleotide polymorphism (SNP) or to the presence of tandem repeat sequences (TRS) have been described. These variable regions are important for phylogenetic studies of ASFV. Comparative analysis of the C-terminal end of the *B646L* gene, which encodes the p72 protein, allows ASFV to be classified into 24 different genotypes (Quembo *et* al., 2019). This method, used internationally, allows relatively quick and easy typing of ASFV strains and remains the first approach to identify the origin of the virus in case of introduction into new territories. However, the genotyping method based on the *B646L* gene, it does not always provide adequate typing resolution or the ability to discriminate between viruses closely related. Analysis of the tandem repeats sequences (TRS) in the central variable region (CVR) of the *B602L* gene or the intergenic region (IGR) between the *I73R* and *I329L* genes at the right end of the genome (Gallardo et al., 2014) can be used to distinguish closely related ASFV isolates. The *B602L* gene is a particularly discriminative genetic marker whose sequencing has distinguished up to 31 subgroups of viruses with varying tetrameric amino acid repeats (Nix *et* al, 2006). Many other gene regions, such as the *E183L, CP204L* and *EP402R* encoding the p54, p30 and CD2v proteins, respectively, have also proved valuable in the analysis of ASFV from various locations to trace its spread (Qu *et* al., 2022).

The complex epidemiological pattern of ASFV is evident in sub-Saharan African that result in greater genetic variability of ASFV isolates from eastern and southern Africa with all 24 genotypes present (Penrith *et* al., 2022). In West African viruses are highly homogeneous, and outbreaks have historically been associated with genotype I, although different studies describe the spread of ASFV genotypes from East Africa to West Africa (Adedeji *et* al., 2021). In 1957, ASF genotype I was identified for the first time in Europe, in Lisbon coming from West Africa (Danzetta *et* al., 2021). In 1960 emerged in Lisbon and after that ASFV spread through the Iberian Peninsula (Spain and other areas of Portugal), and from there to other countries in Europe, the Caribbean and Brazil. Eradication was achieved in the mid-1990s, except in Sardinia, where the disease remains endemic. Genotype I was responsible for this first transcontinental spread.

In 2007, the second jump from the African to the European continent took place, when the ASFV emerged in the

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Republic of Georgia. The cause of this outbreak was a genotype II, which was circulating in Mozambique, Madagascar and Zambia (Rowlands *et* al, 2008). Subsequently, the disease continued to spread through the Caucasus region and later through the Russian Federation and Eastern Europe, until reaching the European Union (EU) in 2014. Since then, ASF has been reported by 13 EU countries, including Lithuania, Poland, Latvia and Estonia (2014), the Czech Republic and Romania (2017), Bulgaria and Hungary (2018), Belgium and Slovakia (2019), Greece and Germany in 2020, and most recently in January 2022, in the Piedmont region of north-western Italy. Two European countries have managed to eradicate the disease: Belgium (event resolved in March 2020) and the Czech Republic (event resolved in April 2018). In August 2018, the worst scenario happened when China reported the outbreak of ASF in the Liaoning province caused by a genotype II strain (Ge *et* al., 2018). Since then, the disease continued to spread in China, and by the end of February of 2022, ASFV was detected in 32 China provinces and in 13 Asian countries, being the latest Thailand in January 2022. In September 2019, the first occurrence of ASF in Oceania was reported by Timor-Leste, followed by Papua New Guinea (March 2020). In July 2021 the disease reappeared in the Americas after an absence of almost 40 years, having been introduced in Dominican Republic and later in Haiti (FAO situation update, www.fao.org).

The investigation of virus molecular evolution in combination with spatio-temporal data is an integral part of pathogen tracing and may help in the identification of potential routes of its spreading, therefore in disease prevention and control. With the introduction of ASF into Asia, place with the highest population of domestic pigs in the world, the world is now facing the worst pandemic of an animal disease seen to date, and new ASFV whole-genome sequences from Europe and Asia are being published with increasing frequency. Currently there are available 139 whole genome sequences from 13 out of the 24 genotypes described, and 41 corresponds to genotype II (figure 1).
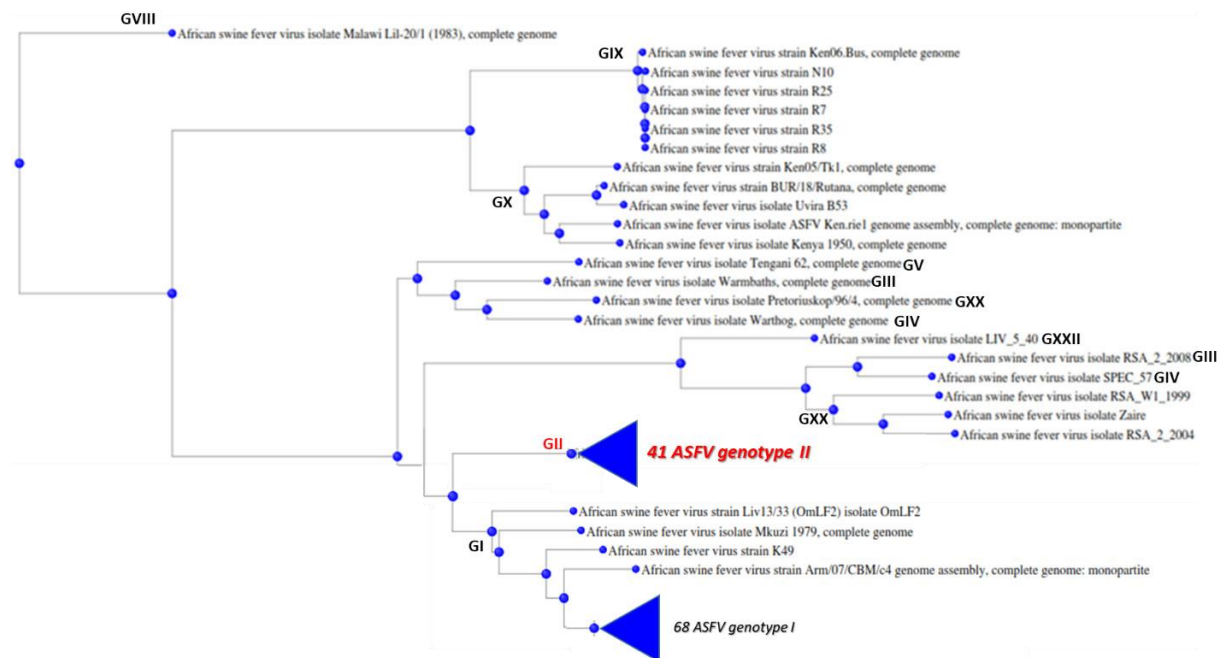
Figure 1. Phylogenetic tree showing all available ASFV complete genome sequences that share a cover > 90% (n=139).

Based on the 41 available whole genomic sequences deposited in the GenBank database, a low mutation rate (< 0.3%) up to date have been detected in the genotype II-ASFVs currently circulating Eurasia and all of them place in the same cluster. In general, ASFV is very stable and this leads to low genetic variability in affected regions and even the use of next-generation sequencing does usually not allow molecular tracking of virus strains in a higher resolution e.g. for molecular epidemiology in an epidemic situation. Published data confirmed that genomic regions containing tandem repeats could reveal disease trajectories in space and time. Due to technical issues, these regions are of particular interest in terms of standard genotyping procedures due to the difference in PCR product length, which is convenient to observe during regular agar electrophoresis. Gallardo *et* al. (2014) found that the sequences of ASFV isolates from index cases in the EU (Lithuania and Poland) had an identical TRS insert (IGR2 variant) to that present in ASFV isolates from Belarus and Ukraine, and different from other viruses in Eastern Europe and Russia. These molecular data, together with epidemiological findings, confirmed that the ASFVs detected in Poland and Lithuania originated in Belarus, but probably emerged in 2012 or even earlier in the Russian Federation. The extensive molecular characterization done at the ASF-EU reference laboratory (EURL, Madrid, Spain) of more than 2,600 ASF isolates from both wild boar and domestic pig obtained between 2014 and 2022 in the EU, identified four different IGR variants (IGR1 to IGR4), with variant IGR2 being the predominant one in the EU and Russia (Mazur-Panasiuk *et* al., 2020). Further analysis of the CVR sequence within the *B602L* gene identified a new ASFV genotype II CVR variant

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2 (GII-CVR2) in southern Estonia in 2015 and 2016 within the wild boar population. The CVR2 variant was characterized by the deletion of three TRSs (Vilem et al., 2020). Similarly, sequencing of the *O174L* gene detected two variants in Poland, with variant 2 characterized by the additional 14 nt insertion, representing a tandem repeat (Mazur-Panasiuk *et* al, 2020). It is important to note that 95% of reported cases in the EU since 2014 have occurred in wild boar populations, and the virus, depending on the ecological context, may persist in wild boar populations with or without reintroduction of infected domestic animals. Therefore, variations in the TRS could be related to a spontaneous mutation caused by the maintenance of ASFV within the wild boar population in certain regions of the EU. Sequencing of the IGR between the *9R* and *10R* genes of MGF505 supported this hypothesis with seven MGF variants identified from wild boar genotype II viruses circulating in Europe (C. Gallardo personal communication 2021).

The virus causing ASFV outbreaks in China was initially classified as genotype II and the IGR-II variant, which is predominant in Europe (Ge *et* al, 2019). Further molecular characterization studies conducted on genotype II isolates from Asia and Oceania since 2018 up to 2022, have identified the four ASFV IGR variants (IGR1 to IGR4) that circulate in both domestic pigs and wild boar in China, Korea and Vietnam. Additional analysis of a new genome marker located between based on the intergenic region between the *A179L* and *A137R* identified multiple genotype II-variants in Vietnam (Tran *et* al., 2021). But without a doubt the most important fact has been the identification of genotype I of ASFV in the Asian continent in July 2021 from pig farms in Henan and Shandong province (Sun *et* al, 2021a). Phylogenetic analysis of the whole genome sequences suggested that both isolates share high similarity with NH/P68 and OURT88/3, two genotype I attenuated ASFVs isolated in Portugal in the last century. It is important to note that animals infected with this virus developed a chronic disease that could go unnoticed in the field due to its reduced virulence. The source of these viruses and the nature of their introduction into China is unclear. Although they may represent a new introduction of the virus from an African source, the striking degree of genetic similarity to NH/P68 and OURT88/3, two genotype I ASFV isolated in Portugal in the 1960, suggests they may have originated from other source, possibly imported legally or illegally for evaluation as potential African swine fever vaccine candidates in China. The emergence of genotype I ASFVs present more problems and challenges for the control and prevention of ASFV in Asia.

In summary, current genotype II ASFV strains affecting Europe and Asia are closely related and share more than 99% homology when whole genome sequences are compared. Introducing a method of subtyping into routine diagnosis within affected areas worldwide, especially new disease incursions, may help identify potential disease origins and provide a deeper understanding of spatial-specific trajectories disease seasons. Due to the low mutation rate of the ASFV genome and its slow molecular evolution, the utility of a single subtyping method within the same genotype is still limited and allows only moderate discrimination of closely related strains. The use of a standardize protocol using multiple genetic markers should be further investigate and implement at international level that may help determine potential disease trajectories with higher resolution. Additionally, since current genetic characterization approaches are not related to biological properties (Arias *et* al., 2018), more research throughout the full ASFV genome length sequence is needed to identify new genetic markers that could explain the moderate virulence and attenuated phenotypes of genotype II-ASFVs. The genetic characterization of the virulence of multigene family (MGF) genes such as the gene MGF505-7R (Li *et* al., 2021) and the EP402R (CD2v) to cluster/group ASFV isolates based on virulence factors could be a potentially interesting area of research.

Biological properties of genotype II Eurasian ASFV strains and its role in the transmission of the disease.

Different strains of ASFV have been found to cause variable clinical presentations, ranging from acute and peracute infections with 90 to 100% mortality, to subacute and chronic forms with much lower mortality (Salguero *et* al., 2020). Genotype II ASFV strains circulating in Europe and Asia are generally highly virulent in both domestic pigs and wild boar, causing acute disease with almost 100% lethality in animals. In experimental infections, after intramuscular inoculation with virulent strains, regardless of dose, animals became infected after an average of 4.4 ± 1.2 days and did not survive for more than 11 days. Previous studies have shown that both the intranasal and oronasal routes are equally lethal, although the nasal route resulted in a higher incidence of ASF than the oral route when a lower infective dose was used. Once infected, animals developed acute clinical signs between 3.5 and 14 dpi (mean) and 91 to 100% of infected animals died between 7 and 21 days after the first case. A similar picture has been observed in pigs when they were exposed to the virulent virus through direct contact with infected animals. Exposed animals developed a similar acute clinic that resulted in death between 11 and 25 days after exposure (Pikalo *et* al., 2021). However, one of the biggest concerns in recent years is the continuing trend towards the appearance of clinically milder or completely unapparent forms in areas where the disease is endemic in Europe and Asia that difficult the control of the disease. This attenuated phenotype was initially reported for a strain in Estonia in 2014 in the northeast of the country where mortality was surprisingly low and anti-ASFV antibodies were detected in hunted animals. *In vivo* studies in wild boar and domestic pigs showed that the ASFV isolated in 2014 in north-eastern Estonia (Ida-Viru region) was moderately virulent in domestic pigs, but remained highly virulent in adult wild boar.

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Genome sequence analysis revealed a 14.5 kilobase pair deletion at the 5-end of the viral DNA, which is responsible for the attenuated phenotype in domestic pigs (Nurmoja *et* al., 2017; Zani *et* al., 2018). One year later, in 2015, Gallardo *et* al. describes the presence of strains of moderate virulence in southern Estonia circulating among the wild boar population (Gallardo *et* al., 2018; Vilem *et*al., 2020). The results obtained from the *in vivo* studies indicated that, regardless of the Estonian ASF strain used, infected pigs presented variable clinical and pathological findings ranging from acute, subacute to chronic forms of ASF, characteristic of viruses of moderate virulence. A 33.3% of pigs survived the infection and exhibited mild, nonspecific clinical signs from approximately 14 days to one month post-infection. After a period of apparent recovery, clinical signs reappeared two months later (50-60 days) and were similar to those described in previous studies using moderately virulent ASFV isolates belonging to p72 genotype I. Similar findings were described by Walczak et al., 2020 using an ASFV from Poland isolated from wild boar (Pol18_28298_O1). Following intranasal infection of domestic pigs with Polish virus, the animals developed various forms of the disease (acute, subacute and chronic) and mortality ranged from 80 to 100% depending on the dose. Two pigs survived the infection with nonspecific clinical signs, no fever, and short viremia.

In 2017 was isolated the first non-haemadsorbing (HAD) and attenuated genotype II ASFV strain, Lv17/WB/Rie1, from a hunted wild boar in Latvia (Gallardo *et* al., 2019). The HAD phenomenon consists of the adsorption of red blood cells around monocytes/macrophages that have been infected by ASFV. The sequence analysis of the *EP402R* gene, coding the CD2-like protein responsible for the ASFV distinctive HAD phenomenon, revealed a single adenosine deletion that generates a truncated protein. In Lv17/WB/Rie1 ASFV isolate, the non-functional CD2-like protein is responsible of its non-HAD capacity, a feature shared with other naturally attenuated ASFV strains, such as NH/P68 and OURT88/3, or the recently discovered non-HAD Chinese genotype I ASFVs (Sun *et* al., 2021a). Pigs experimentally infected with Lv/17/WB/Rie1 ASFV developed non-specific clinical signs, and in some cases remained asymptomatic, showing intermittent and weak viremia and a high antibody response. Furthermore, two months following the primary infection with Lv17/WB/Rie1, the two pigs exposed were fully resistant to challenge with a virulent HAD Latvian ASFV. Since the first description in 2017, eleven non-HAD genotype II ASFVs have been isolated from wild boar in the EU, including Lv17/WB/Rie1 (Gallardo. C, unpublished data). The eleven non-HAD ASFV isolates had different types of mutations or deletions in the *EP402R* gene that prevent the viruses from translating intact CD2v protein and result in a non-HAD phenotype. When tested in domestic pigs induced subacute or chronic diseases, or even some pigs remained asymptomatic. Consistent with what was reported in the EU, 11 non-HAD viruses were isolated in China during a surveillance program conducted from June to December 2020 (Sun *et* al., 2021b). Chinese non-HAD viruses had four different types of naturally occurring mutations or deletions in the EP402R gene and showed lower virulence in domestic pigs, but were highly transmissible similar to that seen with non-HAD ASFVs from the EU.

In conclusion, several studies illustrate the natural evolution of ASFV genotype II in Europe and Asia towards less virulent forms over time circulating together with virulent viruses, as has occurred in other geographic regions where ASF has been present for a long time (Arias *et* al., 2018). Regardless of the genotype responsible for the initial outbreaks, in areas where ASF is not efficiently eradicated, the disease becomes endemic and the virus evolves into moderate and attenuated strains with an increase in the number of subacute, chronic, and subclinical infections. In such situations, the clinical manifestations of the disease are more variable and difficult to recognize in the field. The infection can persist for several months with no particular symptoms evident in infected animals, apart from growth retardation or emaciation, or it can even mimic other diseases. Although a long-term carrier state has not yet been experimentally demonstrated, the question is whether these animals have the capacity to infect a naïve population and whether or not they play an important role in the epidemiology of the disease, that is, in the persistence of the virus in endemic areas, the appearance of sporadic outbreaks and the introduction to new regions. Transmission studies with ASFV genotype II of different virulence have shown that animals surviving acute and subacute infections, shed ASFV by the oral secretions up to 22-30 days and from blood up to about 44-60 days, and that an infected animal could play a role as virus-carrier during that period (Blome *et* al., 2020; Gallardo *et* al., 2021; Walczak *et* al., 2020; Zani *et* al., 2018; Sun *et* al., 2021a,b). Pigs infected with attenuated strains can shed infectious virus from the blood up to about 15 to 20 days, but with titers similar to those of the moderate virulence group. On the contrary, the risk of oral transmission, which is the natural route of infection, is much lower than in the case of infections with strains of high or moderate virulence, although this circumstance cannot be excluded. In fact, Gallardo *et* al., (2015) reported that seropositive animals infected with genotype I NH/P68 ASFV were able to transmit the virus to a susceptible population, more than three months after the first virus inoculation, even in the absence of viremia or clinical signs. Since the virus was isolated from the lung and mediastinal lymph nodes four and half months later, the ASFV could be easily transmitted from the respiratory tract through oral excretions.

These results contradict the assertion of some researchers that the probability of a seropositive but virus-negative "survivor animal" shedding infectious virus and playing a role as a carrier is practically zero. It cannot be excluded that a very small number of animals can transmit ASFV even in the absence of virus presence in blood and thus maintain the virus in endemic areas. An example could be found in Estonia. In this country, wild boar that were ASFV-positive, seropositive, or both, were regularly detected until February 2019. Thereafter and for more than 1 year, only

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wild boar were found to be seropositive but negative to ASFV and no outbreak had been detected in domestic pigs since 2017. Since August 2020, several ASFV-positive wild boar were reported in the centre and north-east of the country and the virus appeared in a domestic pig farm in 2021. The re-emergence of ASFV-positives wild boar and domestic pigs raised the question of the role of seropositive wild boar in the epidemic situation in the country. Schulz *et* al (2021) hypothesized that the most likely reason for this re-emergence was the reintroduction of ASFV from neighbouring countries. However, the study by Schulz did not exclude that seropositive but virus-negative wild boar are capable of transmitting ASFV and spreading the disease. In addition, an important factor to take into account is the type of samples that are analysed in epidemiological surveillance programs. Both the matrix used and the quality of the samples may influence the probability of detecting low amounts of viral genomes or that the virus is confined in a specific tissue. In this respect, screening of various tissues could aid detection of potential virus-carrier. The experimental *in vivo* studies with ASFV genotype II strains of different virulence, reaffirms this statement. In surviving animals and those that develop chronic or subclinical infections, the virus is cleared more or less rapidly from target organs such as bone marrow, spleen, or kidney. However, it persists for more than two months, even up to four months, in primary replication sites, such as tonsils and lymph nodes, or in secondary replication sites, such as intra-articular tissues (Gallardo et al., 2019; 2021; Walczak et al., 2020). This long-term detection of virus in animals infected with attenuated and moderately virulent isolates has been described in previous studies with ASFV genotype I viruses and virus was detected in lymph nodes and/or tonsils for long periods of time, even up to 13 weeks after infection (Gallardo *e*t al., 2015). The localized presence of virus in lymphoid tissues, primary replication sites, occurring to some extent in any of the survivor categories, could suggest the likelihood of persistent infection or that pigs have multiple reinfections with the same strain, as virus is usually present where primary viral replication occurs. All together these data suggest that other tissues should be also considered as target samples in the surveillance programs. Furthermore, the question whether the ASFV present in a tissue could be reactivated in seropositive wild boar under immunosuppression, stress or in case of death has to be pursued further. To find scientific evidence regarding these questions, it will be inevitable to conduct long-term experimental studies.

ASF diagnosis.

State of the art, gaps and priorities

Since there is no vaccine available, prevention, control, and eradication of ASF is based on the implementation of appropriated surveillance that detects ASF outbreaks as early as possible, as well as the ability to respond to outbreaks quickly and efficiently so that ASFV spread can be prevented and, ideally, eradicated. A key element of ASF control strategies is the early detection of infected domestic and wild pigs. This is important for any infectious disease, but even more so for ASFV, because the virus survives for extended periods in the environment and in pork products, and because the appearance the presence of ASF of different virulence co-circulating in affected countries regardless the genotype is affecting. Therefore, any onward spread prior to detection will have a major adverse impact on the ability to contain or stop spread. The design of a sufficiently sensitive ASF surveillance system requires a sound understanding of the epidemiology, the virus, and the disease, coupled with adequate diagnostic laboratory infrastructure with qualified personnel, adequate financial resources, and internationally validated techniques. Both passive (observer-initiated) and active (investigator-initiated) surveillance system components may be used, but the passive component is of major importance for early detection in domestic and wild pigs. Passive surveillance is based on farmers, other actors involved in the pork food system, and anyone encountering potentially diseased wild pigs notifying the veterinary authorities of their suspicion. Active surveillance implies actively looking for infected or clinically diseased domestic and wild pigs and sampling legal and illegal live pig and pork imports at border inspection posts.

Success of surveillance activities depends on the availability of the most appropriate diagnostic tests. A wide spectrum of accurate ASF diagnostic tests is available and most of them have been successfully employed in surveillance, control and eradication programs (Gallardo et al., 2019b). However, as in any other disease, there is not a single test being 100% reliable (sensitive and specific). For this reason, final diagnosis should be based on the interpretation of the results derived from the use of a number of validated tests in the appropriated samples, in combination with the information coming from disease epidemiology, scenario, and the clinical signs.

ASF diagnostic workflow

In case of an ASF suspicion, the PCR is by far the most sensitive method for the detection of the agent and the method of choice for first-line laboratory diagnosis. It is a basic diagnostic tool for surveillance, considering the long-term viremia, the high viral load in the infected animals suffering acute or subacute clinical courses. It is quick and can be used for individual as well as pooled samples although with size-limitation (Gallardo et al., 2019b). A variety of PCR tests, including both conventional and real time (rtPCR), as well as commercial kits have been developed and validated

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to detect a wide range of ASF isolates belonging to different known virus genotypes, non-HAD strains, and diverse virulence (Auer *et* al, 2022; Gallardo *et* al., 2019b; Pikalo *et* al., 2022). Nevertheless, although rare, to avoid any false positive PCR results, (e.g., due to lab contamination or other factors) several procedures are implemented. Thus a primary outbreak (or wild boar case) of ASF should be ideally confirmed by virus isolation of ASFV and the identification by the HAD assay, by the national reference laboratories or at international level, and by genetic typing at the laboratories. A recent described duplex real-time PCR based on ASFV E296R gene for rapid detection and differentiation between genotypes I and II ASFVs (Li et al., 2022) provides an additional powerful tool that can facilitate efficient control of ASFV in regions where both genotypes can be circulating such as China or several African countries (i.e. Nigeria). However, this might not always be possible due to technical limitations, absence or appropriated facilities or the reduced sensitivity, particularly in samples obtained from altered carcasses or hunted wild boar, or in weak positive PCR samples (Gallardo *et* al., 2019b).

Whenever the suspicion is raised that ASFV is circulating in a swine population, a negative PCR result cannot excluded the presence of ASF. Since animals usually develop antibodies within the second week after infection, they can test positive for both ASFV and antibodies simultaneously for at least two months. Samples from animals surviving this period are usually positive for ASFV-specific antibodies, but negative for ASFV and its genome. Therefore, if the PCR gave a negative result but there is a suspicion that ASFV is circulating, serological assays should also be used for the diagnosis. The current recommendations for ASFV antibody detection involve the use of an ELISA for antibody screening, backed up by Immunoblotting (IB), Indirect Immunofluorescence test (IFAT) or the Indirect immunoperoxidase tests (IPT) as confirmatory tests (OIE 2021). The ELISA remains the most useful methodfor large-scale serological studies in serum samples: it is fast, easy to perform and economical. However, only serumcan be analysed, which restricts its application range, especially in case of passive surveillance of wild boar when animals are usually found dead. In addition, hemolysed serum samples could arose either false positive or negative results depending of the ELISA format employed. Therefore, positive ELISA results should always be confirmed by additional methods such as IPT, IFAT or IB tests, as recommended by the OIE (OIE 2021). The IB is a rapid and sensitive assay but, similarly to that described above, only serum samples can be tested. On the contrary, IPT or IFAT can be easily used for analysing all type of porcine samples, including exudates from tissue, whole blood, fluids and even bone marrow. The antibody detection by IPT in exudates tissue samples is a common successful method when wild boar are analysed.

Taken together, sensitive, specific and robust laboratory diagnostic assays are available but, as for any other disease, there is not a single test being 100% reliable (sensitive and specific). For this reason, final diagnosis should be based on the interpretation of the results derived from the use of appropriate samples and validated tests in combination with the information coming from disease epidemiology, the presence of clinical signs and the scenario. A thorough understanding of the viremia and antibody seroconversion timing during ASFV infection is a prerequisite to conclude the dynamic of the infection in the investigated areas, and to support control and eradication programs. Positive results for both virus and antibodies indicate that the tested animal was infected at the time of sampling, whereas a positive ASFV antibody test in absence of virus indicates an ongoing or past infection, where the animal has recovered or could be chronically or sub-clinically infected with attenuated strain. These animals should be detected since they can act as carrier of the virus and, in certain conditions to infect a naive population

On the international level, laboratory methods as well as sampling and shipping guidelines can be found in the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 3.9.1, version adopted in May 2021). The selection of which test to use depends on available matrices, the purpose of the testing (surveillance, eradication, diagnosis, confirmation), as well as the ASF epidemiological status of the country (region) or stage of the epidemic in the region.

Sampling: classical versus alternative sampling methods

The starting point for any laboratory investigation on ASF is sample collection. An important consideration is the purpose of the investigation, for example disease diagnosis, disease surveillance, or health certification. Which animals to sample will depend on the objective of the sampling. For example, when investigating an outbreak (passive surveillance), sick and dead animals should be targeted, while the oldest animals should be sampled when checking if animals have been exposed to the disease (active surveillance). To be effective, appropriate samples combined withthe selection of diagnostic methods are of fundamental importance in order to make a rapid and reliable diagnosis. Samples collected from live pigs should include anti-coagulated whole blood for the detection of virus or viral nucleic acid and serum for the detection of antibodies, whereas samples collected from dead pigs or wild boar should comprise tissues for both virus and antibody detection (table 1)

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Table 1: target samples for ASF virus and antibody detection, the classical ASF diagnostic tests and their recommended use.

DETECTION	TECHIQUE	TARGET SAMPLES	RECOMMENDED USE
Nucleic acid detection	PCR tests (i.h. conventional and real time PCR tests and commercial tests)	**Organs:** spleen, lymph nodes, liver, tonsil, heart, lung, kidney, bone marrow (wild boar) and intra-articular cartilage.	Early detection: suspicion, outbreak investigation, surveillance.
		Anticoagulated **blood*** **Ticks**	Individual and herd testing. Movements from restricted zones
Virus detection	Virus isolation and identification by haemadsorption (HAD) test (i.h)	**Organs:** spleen, lymph nodes, liver, tonsil, heart, lung, kidney, bone marrow (wild boar) and intra-articular cartilage. Anticoagulated **blood*** **Ticks**	Confirmation of primary outbreak.
Antigen detection	Direct Immunofluorescence (DIF) (i.h)	**Organs:** spleen, lymph nodes and tonsil.	Individual and herd testing (in case of clinical signs), early detection. *It is recommended its use in parallel with antibody detection tests.*
	Antigen ELISA commercial kit INgezimPPA DAS, Double Ab Sandwich.	**Organs:** spleen and lymph nodes. **Plasma** from anticoagulated blood	Surveillance Herd testing (in case of clinical signs).
Antibody detection	ELISA (i.h ELISA tests and commercial methods)	**Sera**	Individual and herd testing when deemed appropriate. Surveillance
	Immunoblot (IB) (i.h)	**Sera**	Confirmatory test Individual and herd testing when deemed appropriate.
	Indirect Immunoperoxidase test (IPT) (i.h)	**Sera** **Plasma** from anticoagulated blood **Exudates from tissues** **Corporal fluids** (pericardial, intraarticular, thoracic, etc)	Confirmatory test Individual and herd testing when deemed appropriate. Surveillance; epidemiological studies (time of the infection)
	Immunofluorescence Antibody (IFAT) test (i.h)	**Sera** **Plasma** from anticoagulated blood **Exudates from tissues** **Corporal fluids** (pericardial, intraarticular, thoracic, etc)	Confirmatory test Individual and herd testing when deemed appropriate. Surveillance; epidemiological studies (time of the infection)

Source: webpage of the ASF-EU reference laboratory (EURL); https://asf-referencelab.info/asf/en/

The sample matrices described above are routine for veterinary practitioners or pathologists. However, these sample types may not always be available and alternative samples may be better suited, especially for wild boar carcasses and for active ASF surveillance in large pig farms. Different types of alternative samples have been tested that meet the objective of providing a reliable diagnosis. Among the published options are dry blood swabs, dried filter papers and FTA cards, fecal samples, oral, nasal and rectal swabs, meat-juice, different rope-based options, ear punches or dry-Sponges (manufactured by 3M) (Flannery *et* al., 2020; Kosowska *et* al. 2021; Onyilagha *et* al., 2021; Pikalo *et* al., 2021). This review summarizes the latter approaches that has provided reliable results.

While shedding will depend on the virulence of the isolate most secretions and excretions will be positive for ASFV genomes in the clinical phase, although some considerations should be taken into account. (Gallardo *et* al., 2021). The ASFV through the feces occurs only in the acute phase of the infection caused by virulent strains and two or even four days later than in blood, therefore, the use of feces or rectal swabs seems to be limited in the diagnosis of ASF. After the acute phase, the presence of the virus in the stool decreases rapidly, making it an unreliable diagnostic sample. In addition, it must be taken into account that the half-life of the virus in the field is strongly affected by the enzymes (proteases and lipases) produced by bacteria that colonize the feces, so the survival of the virus in the field is not comparable with the estimates obtained under laboratory conditions (EFSA 2018). In contrast, the ASFV genome could be then easily detected by PCR from oropharyngeal swabs earlier than in blood, independently of the strain virulence. This is due to primary replication in the tonsil and retropharyngeal lymph nodes in the normal route of infection in the field. However, during the course of the infection, important differences are detected depending on the virulence of the strain and the clinical form of the disease. When samples are collected within the first three weeks of infection (≈ 3-20 days), the highest proportion of PCR-positive samples is obtained from oropharyngeal swabs, regardless of strain. On the contrary, in the blood samples there are significant differences. While in acute and subacute

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infections the virus is detected in similar proportions in blood and in oropharyngeal swabs, in animals with chronic or subclinical disease, viremia peaks are intermittent, even at the beginning of the infection. As of day 20, the ASFV genome is only detected sporadically in these animals and in a clearly lower percentage than that detected in oropharyngeal samples (Gallardo *e*t al., 2021). However, in animals that survive infection caused by moderately virulent isolates, the ASFV genome can be detected in blood for a period of about two months or even up to 100 days (Blome *et al.*, 2020), while in oropharyngeal samples the detection range is usually lower at one month (Gallardo et al. 2021). Therefore, the oropharyngeal swabs should not be used as a substitute for blood in active surveillance, as it would decrease the detection of animals that survived to the primary infection with either virulence or moderate virulence ASFVs, as they would possibly not be showing any clinical signs. But together with blood, the oropharyngeal swabs samples could allow to detect ASFV infection for a longer period and could be a useful an alternative sample in the passive surveillance programs for the early detection prior to onset of obvious clinical signs, mainly in large pig farms (Gallardo *et al.*, 2021; Pikalo *et al.*, 2021). Regarding the detection of antibodies, more studies are needed since, compared to serum, the sensitivity percentages are below the adequate limits to give a reliable diagnosis (Gallardo C. personal communication 2021).

Sampling individual pigs on commercial pig farms is a cornerstone of current surveillance for ASFV, but it is labour-intensive and expensive. Rope-based oral fluid collection is a non-invasive method that is widely used in industry as a diagnostic and surveillance sample to detect various endemic swine pathogens. Collection of oral fluids with ropes in a pen can be performed by non-veterinary personnel with minimal resources and discomfort to the animals. In recent experimental studies using domestic pigs inoculated with highly virulent ASFV Georgia 2007/1 or moderatelyvirulent ASFV Malta' 78, the ASFV genome was detected in oral fluid before the animals developed noticeable clinicalsigns and the pigs continued to chew the ropes daily until severe clinical signs developed (Goonewardene *et* al., 2021).However, the ASFV genome was detected in oral fluids at low to moderate levels (Ct > 30) and between 2 and 4 dayslater than in blood. These results, consistent with that described by Lee *et* al., 2021 using a virulent strain from Vietnam, suggest that the use of oral fluids to supplement the use of traditional samples for rapid detection during ASF surveillance should be carefully considered and requires more field validation studies. When it comes to antibodydetection, oral fluids were shown to work with a slight delay in detection and depends of the type of antibody test used (Mur *et* al., 2013).

Over the last years, the blood swabs as an alternative matrix for passive surveillance, especially in wild boar, has been widely used, even for field detection (Sauter-Louis *et* al., 2021). However, sensitivity is dependent of several factors such as the circulating strains and the type of swabs and buffers used (Gallardo C. personal communication 2021; Pikalo *et* al., 2021). In a recent study conducted at EURL-ASF (Madrid, Spain), a total of four hundred and sixteen EDTA-blood samples from experimental and field infections were used for routine PCR testing and dried blood swab generation using cotton devices. Animals were infected with ASFV genotype II strains of different virulence, including the attenuated ASFV Lv17/WB/Rie1. Dried blood swabs were tested in parallel using the OIE real-time PCR developed by Fernandez Pinero *et* al., 2013 (OIE 2021) and the IPT for virus and antibody detection (OIE 2021).Using the PCR test, the percentage of agreement was 90% between blood and swabs when samples had Ct <30. However, the percentages decreased dramatically (<35%) when testing samples with Ct >30. This was related to the time of infection and the clinical form. False negative results in swabs were obtained early in the infection but especially in animals that had a chronic or subclinical type of infection where viremia is usually weak. The paired swabs and blood were then tested by IPT for antibody detection. Sensitivity was 78% with 32 false negative results inswabs. Combining the results of PCR and IPT, the percentage of positives in blood was 91% compared to 81% in swabs, depicting almost a perfect agreement [□ =0.9 $_{95\%CI}$] between both type of samples. In summary, of the 416 samples from diverse source, only 18 cases (4.3%) would not have been identified as infected using the blood swabs.Other studies reported even increased sensitivity when Genotubes (Carlson *et* al., 2018) or PrimeSwab are used (Pikalo*et* al., 2021), so fast-drying swabs could be an alternative for ASF detection. They have several advantages from easyhandling to long-term storage and the ability to cut and use one swab for multiple diagnostic tests. Another key feature of this swab is the diversity of samples it may be used with, including organs and bone marrow from dead wild boar.In conclusion, swabs are a practical, inexpensive and straightforward approach for passive surveillance of ASFV, mainly in the deceased wild boar. Whether it is worth using this approach instead of classical sampling system, remainsthe choice of users based on risk assessment, integration into surveillance strategies, and financial resources.

The use of blood samples dried on filter papers has been also described as a possible alternative to for serological and virological testing. Whatman FTA cards consists of filter papers specifically engineered for nucleic acid preparation and preservation. They contain impregnated matrices that lyse cells, denature proteins and protect nucleic acids from nucleases, thus providing additional useful inactivation of the biological material which theoretically makes it compatible with safe shipment without the need for containment. However, in the laboratory, they require extra-preparation such as rinsing and elution before being used for diagnosis and are not suitable for subsequent pathogen isolation nor by antibody detection. Other filter papers like Whatman 3-MM filter papers do not contain additives; they can thus preserve infectivity and can be used for both virus and antibody detection. However, dried blood samples on filter paper provide variable results depending on the diagnostic method used and are not versatile samples that can

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be analyzed in all laboratories (Randriamparany *et* al., 2016). Finally, although with certain limitations, ear tissue samples and meat juice, especially the diaphragmatic muscle, has proven to be a good matrix to the detection of ASFV and/or ASFV specific antibodies (Onyilagha *et* al., 2021)

In summary, the comparative studies of alternative samples *vs* classical samples confirmed that EDTA blood is the most suitable option for ASFV genome and antibody detection, together with sera, both in the initial phase of infection as in late. Alternative samples, such as oropharyngeal or blood swabs, have shown to be the most promising alternative samples and could detect ASFV and/or antibodies to some extent, although sensitivity results depend on virulence of the strain. In conclusion, alternative approaches are feasible, but should be integrated into control strategies by selecting test methods and sample materials following a "fit for purpose" approach.

ASF diagnosis; gaps and future trends

A large number of validated ASF diagnostic techniques are now available to provide a reliable diagnosis of ASF in affected countries (Gallardo *et* al., 2019b). However, there are some gaps that have begun to be filled in the last five years. This section will discuss some of the advances made in the development of rapid, reliable, sensitive, and convenient diagnostic tests with the potential to overcome the limitations of currently available assays.

Molecular tests

There is a strong demand for accurate, rapid, and simple detection methods especially for on-site application. Nucleic acid testing is the most commonly used method for ASFV detection. However, traditional nucleic acid purification step is time- and labor-consuming. The nucleic acid purification, amplification and amplicons detection rely on laboratory settings which limits the on-site detection. Point of care (PoC) molecular detection methods have been adapted for ASFV genome detection and preliminary validation has been achieved although with limit number of samples (Gaudreault *et* al., 2020; Zurita *et* al., 2021). Other affordable diagnostic solutions include the isothermal assays that could be a cheaper diagnostic alternative to PCR, and useful in field conditions. Numerous studies from recent years have described new diagnostic tests based on this technology, such as recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP) and cross-priming amplification (CPA). Moreover, those isothermal amplification assays in combination with immunochromatographic strips have also been developed for application in the field. The main drawback of these techniques is the lack of high sensitivity which limits their application in the detection of ASFV. Recently, nucleic acid detection techniques based on the clustered regularly interspaced short palindromic repeats (CRISPR)-associated endonucleases (CRISPR/Cas) systems have been developed. The detection relies on the cleavage preferences of Cas12 or Cas13 in a nonspecific way after binding to a specific target DNA or RNA via programmable guide RNAs. Combined with isothermal amplification RPA assay, the CRISPR system has been used for detecting ASFV. However, the high cost of RPA assay limits its application in the field. A recent study (Yang *et* al., 2021) describes a LAMP assay coupled with the CRISPR)/Cas12a system established in one tube for the detection of the ASFV p72 gene. The performance of the LAMP–CRISPR assay was compared with the OIE real time PCR test (OIE 2021) testing 41 clinical samples including nasal swab, spleen, liver, lung, submandibular lymph node and kidney. The result showed that these two assays had 96.6% consistency, which supports the fact that the LAMP–CRISPR could be regarded as a novel diagnostic assay for the detection of ASFV. The method shed a light on the convenient, portable, low cost, demonstrating a great application potential for monitoring on-site ASFV in the field. However, although depicting rapid result and good specificity and sensitivity, the low number of samples tested in this study limits its use for giving a reliable and confident diagnosis and further validation is still required to better understand the reliability and utility of the test as ASF diagnostic method.

In conclusion the are several approaches for rapid nucleic acid detection, including molecular platforms now available that could allow sensitive ASFV DNA detection in infected pigs, mainly at the early stages of disease. These tests can also be used to detect contaminated carcasses, and pork and environmental samples at the point-of-need (e.g. abattoir, airport or wild boar/feral pig habitats). However, these platforms are technically more complex than rapid antibody or antigen tests and require further field validation studies and a much higher level of training and competency for accurate testing. Molecular field tests also require expensive equipment for amplification and, in many cases, for extracting viral DNA. An update of the current knowledge of the OIE ASF Reference Laboratory Network on commercially available molecular point of care (PoC) tests, including a range of technical details, cost, as well as advantages and disadvantages of each is available at https://www.oie.int/app/uploads/2022/02/2022-02-09-final-oie-asf-tests-guide.pdf.

Antigen detection tests

The only commercially available ELISA test is the INgezim PPA DAS 2.0 (INGEGASA, Eurofins Technologie), but has low sensitivity and is only recommended for herd tests and always together with other virus or antibody detection

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techniques (SauterLouis *et* al 2021; Gallardo *et* al. 2019b). Despite not being included in the Register of Diagnostic Kits certified by the OIE as validated as fit for purpose, there are several PoC tests, that are available commercially for field testing, including basic rapid test kits for detecting antigens using lateral flow devices (LFD). These tests are simple to use, require minimal training and can provide a result within approximately 20 minutes. Rapid antigen tests are typically less sensitive than molecular techniques for virus detection, but some can have comparable levels of specificity. Antigen tests are recommended for use on symptomatic and terminally ill pigs that have high levels of viraemia, rather than on pigs in the early stages of clinical infection that may not have high enough viraemia to allow detection. It is recommended that samples from more than one sick pig are tested to increase the chances of detecting infection (table 2).

Table 2: comparison of four major PoC test methods for rapid ASF virus antigen detection

Test	Ingenasa	Bionote	PenCheck™	Shenzhen Lvshiyuan Biotechnology Co.
Catalogue no.	INgezim ASF CROM Ag (11.ASFV.K.42)	Anigen ASFV Ag rapid test (RG1407DD)	Rapid Screening Test for ASFV (PC-888)	SLB ASF antigen detection RDT
Specimen Type(s)	Whole blood	Serum, plasma or whole blood	Whole blood	Whole blood
Format	Lateral flow	Lateral flow	Dipstick	Lateral flow
Level of assessment	Peer-reviewed published journal article / Independent assessment at reference laboratories	Independent assessment at reference laboratories	Independent assessment at reference laboratory	Peer-reviewed published journal article / Independent laboratory assessment
Sensitivity	Low to moderate (~68%)	Low to moderate*	Low*	Low to moderate (~65%)
Specificity	High (98%)	Moderate*	Moderate to high*	Moderate (~76%)
Training	Low	Low	Low	Low
Testing Time	15 min	20 min	25-30 min	15-20 min
Cost/test (USD)	$5.80 to $10.45 (depending on pack size)	$14	$2,50	$3,50
Cost of equipment	None	None	None	None
Advantages	Rapid (early detection at POC) / Easy (anyone can perform) / Inexpensive / No equipment costs / High specificity	Rapid (early detection at POC) / Easy (anyone can perform) / Inexpensive / No equipment costs	Rapid (early detection at POC) / Minimal training (e.g. pipette use) / Inexpensive / Minimal equipment required (pipette and tips for aliquotting test reagent) / Moderate to high specificity	Rapid (early detection at POC) / Easy (anyone can perform) / Inexpensive / No equipment costs
Disadvantages	Sensitivity low to moderate, but high enough for testing very sick and dying animals	Sensitivity low to moderate, but high enough for testing very sick and dying animals; moderate specificity (--> false positives)	Low sensitivity	Sensitivity low to moderate, but high enough for testing very sick and dying animals; moderate specificity (--> false positives)
References	Sastre et al. (2016a)	Peer-reviewed publication not yet available	Peer-reviewed publication not yet available	Matsumato et al. (2020)

Source: The OIE ASF Reference Laboratory Network's overview of African swine fever diagnostic tests for field application. Authors Ken Inui, Carmina Gallardo, Raquel Portugal, Linda Dixon, Carrie Baton & David Williams (https://www.oie.int/app/uploads/2022/02/2022-02-09- final-oie-asf-tests-guide.pdf)

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Virus isolation and identification by the HAD assay

Several established cell lines, such as IPAM, COS-1, and WSL, have been used to propagate and titrate limited strains of ASFV, but are not suitable for isolation of ASFV from field samples without little prior adaptation (Gallardo *et* al., 2019b). Currently, virus isolation from field samples relies on primary cell cultures such as porcine lung alveolar macrophages (PAM) or peripheral blood monocytes (PBM). This procedure is more expensive than other techniques, requires both specialized facilities and training, is time consuming and cannot be adapted to high throughput. Therefore, to find an established cell line with potential use in ASF diagnosis is strongly needed. One such cell line was identified in 2020, when Rai et al. reported the successful use of MA-104 cells (a commercially available African green monkey kidney epithelial cell line) for the isolation of several infectious strains of ASFV (Rai *et* al. 2020). The sensitivity of this test was found to be ~10-fold lower than with primary porcine macrophages, but ~10-fold higher than that of a qPCR assay. Importantly, MA-104 cells infected with HAD isolates were also found to exhibit HAD in the presence of porcine erythrocytes, and cells infected with non-HAD isolates were identified by immunostaining. Preliminary studies also showed that ASFV was isolated from infected blood samples, indicating that MA-104 is also a good substrate for direct isolation from field samples without the need of prior passage in primary cells (Rai *et* al. 2020). Ray et al. later published a detailed protocol describing infection of MA-104 cells for detection and quantification of infectious isolates by HAD assays or immunostaining (Rai *et* al. 2021). Since ASFV primarily infects macrophages and monocytes, numerous efforts have been made to establish immortalized cell lines of these lineages to avoid genetic changes that may arise after passage in monkey cells. It has recently been shown that different immortalized primary porcine macrophage cell lines such as IPKM (Masujin *et* al, 2021) or ZMAC-4 (Portugal *et* al, 2020) are capable of effectively replicating different ASFV isolates. However, these cells require further investigation to verify whether the virus is isolated directly from clinical samples, without adaptation process, and maintain a productive viral replication. Therefore, although great progress has been made in recent years on this topic, further studies are needed to achieve the goal of having an established cell line for ASFV diagnostic purposes. These studies should be aimed at validating the recently described cell lines with clinical samples and their suitability for isolating ASFV without inducing genetic and/or phenotypic changes.

Antibody detection techniques

The ELISA test is the most widely used test to detect antibodies on a large scale. The available ELISAs against anti-ASFV antibodies, although generally very specific and sensitive, are only suitable for serum samples, which limits their applicability, especially in endemic areas that lack standardized wild boar sample collection programs (Gallardo, *et* al., 2019b). This issue is nowadays surpassed by the use of IPT test, which can easily analyze all type of samples such as blood and exudates from tissue samples, including bone marrow. However, the IPT requires the use of fixed cultured VERO or MS monolayer cell lines infected with adapted ASFV, therefore needs special biosafety conditions and the interpretation of the results can be subjective and well-trained staff is required. The major drawback is that this technique is not produced commercially by companies, which constrains its use in laboratories, especially those with limited resources. In this context, standardized ELISAs are needed for the detection of specific antibodies in tissue extracts or blood for an easy and more reliable evaluation of epidemiological situation in affected areas. Different ELISAs has been validated for the use of dried blood-spots (DBS) on filter papers with good specificity and relatively appropriate sensitivity (Giménez-Lirola *et* al. 2016; Randriamparany *et* al., 2016). This matrix obviates the need for a cold chain to preserve specimens during the transport to the laboratories and requires only a small sample volume, and needs minimal technical expertise for collecting. Commercial kits, evaluated/validated for use with meat exudate, can be used to detect antibodies to ASFV-proteins in meat exudate samples in order to obtain epidemiological information related to low and moderately virulent ASFV strains circulating in wild boars and domestic pigs, thereby facilitating ASF control and business continuity. Different studies have evaluate the suitability of meat, blood and/or tissue exudate as an alternative sample type for ASF serological detection (Gallardo *et* al., 2021; Onyilagha *et* al., 2021). The INgezim® ASFV-R ELISA technique, which uses a monoclonal antibody (Mab) specific for porcine IgG and recombinant proteins cp312 and p30 of ASFV, has been validated for the detection of specific antibodies in serum, blood (fresh or on paper) and spleen exudate samples from pigs and wild boars, with blood being the best target sample. Due to the lower sensitivity detected in acute infections when the antibody titer is ≤1:2560, the test should be used in parallel with an antigen detection test, to complement surveillance programs in endemic areas. But, in general, these results indicate that anti-ASFV antibodies can be detected in tissue and blood samples using the ELISA format, when sera will not be available in case of dead animals. This is especially interesting in endemic areas where strains of low virulence circulate and viruses in organs are not easily detected. These animals have a high titer of antibodies in the tissues (Gallardo *e*t al., 2018, 2019a, 2021) and therefore could be easily detected by the ELISA test, providing a more detailed overview of the epidemiological situation in endemic areas.

For detection in the field, developed rapid antibody tests (LFDs) generally have comparable levels of sensitivity and specificity to laboratory ELISAs, although they exhibit lower sensitivity compared to reference tests such as IPT. LFDs can be used to detect antibodies in pigs that have survived infection although, as with LFD antigen, both types of tests should be used in parallel to avoid losing infected animals (table 3).

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Table 3: reviews three major PoC test methods for rapid ASF virus antibody detection.

Test	Ingenasa (ASFV/CSFV duplex)	Ingenasa (ASFV)	Global Dx
Catalogue no.	INgezim ASFV-CSFV CROM Ab (11.SFV.K41)	INGEZIM PPA CROM (11.PPA.K41/25)	GDX70-2 Herdscreen® ASF Antibody
Specimen Type(s)	Whole blood and porcine serum samples	Whole blood, plasma, and porcine serum samples	Swine whole blood, plasma or serum
Format	Lateral flow	Lateral flow	Lateral flow
Level of assessment	Peer-reviewed published journal article Independent assessment at reference laboratories	Peer-reviewed published journal article Independent assessment at reference laboratories	Independent assessment at reference laboratories
Sensitivity	Moderate to high (CSFV-92%/ASFV-87%)	Moderate to high (82% sensitivity with respect to the immunoperoxidase monolayer assay [IPMA] in wild boar; 99% correspondence to ELISA)	Moderate to high analytical Ss. (Correspondence with IPMA is 86.2%. Equivalent or higher sensitivity than the commercial ELISAs.)
Specificity	High (98.4%-CSFV/ASFV-100%)	High (99.9% correspondence with ELISAs. 96% specificity respect IPMA [wild boar])	High (100% correspondence with reference technique IPMA)
Training	Low	Low	Low
Testing Time	15 to 30 min	15 to 30 min	15 to 30 min
Cost/test (USD)	$16,38	$5.43 to (depending on pack size)	$4,80
Cost of equipment	None	None	None
Advantages	Rapid (early detection at POC) Easy (anyone can perform) Inexpensive No equipment costs Differential diagnosis of CSFV-ASFV	Rapid (early detection at POC) Easy (anyone can perform) Inexpensive No equipment costs	Rapid (early detection at POC) Easy (anyone can perform) Inexpensive No equipment costs
Disadvantages	Moderate diagnostic sensitivity for ASFV antibody detection. It is recomended to use in parallel with the Ag LFA	Moderate diagnostic sensitivity for ASFV antibody detection. It is recomended to use in parallel with the Ag LFA	Requires further field validation
References	Sastre et al. (2016b).	Cappai et al. (2017).	Peer-reviewed publication not yet available

Concluding remarks

ASFV is a complex DNA virus that has a significant impact on the global swine industry. The lack of a safe and effective vaccine and the reliance on herd culling to prevent the spread of the disease has resulted in significant economic losses. Therefore, improved early detection remains a significant priority. Despite the great effort made in the last five years, there are still some gaps to fill. The low rate of variation of the African swine fever virus genome and its enormous size make it difficult to properly type newly emerging African swine fever virus isolates and thus make it difficult to trace outbreaks. An international effort should be made to develop a standardized genotyping method based on multiple loci of the ASFV genome to identify the origin of outbreaks. The trend towards endemicity of ASFV in the affected regions of Europe and Asia increases the presence of less virulent strains that induce non-specific clinical signs and make it difficult to recognize the disease in the field. It is necessary to increase knowledge about the mechanisms of spread and persistence of ASF in endemic areas and elucidate the role of animals that survive the disease, that is, the role of seropositive animals as potential carriers based on diagnostic data. These data are essential to determine the dynamics of the infection in the affected countries and support control and eradication programs. The use of alternative samples, such as blood or oropharyngeal swabs, can support these programs. While these alternative approaches are feasible, they should be integrated into control strategies through the selection of test methods and sample materials following a "fit for purpose" approach. Finally, since there is a strong demand for the development of accurate, fast and simple detection methods, especially for in situ application, significant efforts should be made to validate them in the field at an international level.

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Wild boar/feral pigs and African swine fever: the management options

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Introduction

The first African Swine Fever (ASF) panzootic started in Portugal in 1957 and expanded to several continents. Many European countries were affected until ASF became finally eradicated from mainland Europe by 1995, with only the Italian island Sardinia remaining infected (Costard et al., 2013). During this period, ASF virus spilled over into the native Eurasian wild boar (*Sus scrofa*). However, despite these spill-over events, ASF eradication succeeded after acting only on domestic pigs, essentially through movement bans and depopulation (Mur et al., 2012).

The second and still ongoing ASF panzootic started in Georgia in 2007 spreading both westwards into Europe (Cwynar et al., 2019) and eastwards into Asia and Oceania (Jo & Gortázar, 2020). In 2022, ASF also reached La Española Island in the Caribbean, adding one more continent to the expanding ASF distribution. During this second panzootic, wild boar do play a prominent role in ASF spread and maintenance (O'Neill et al., 2020), and ASFV infected wild boar are regarded as a significant threat to pig farming where both forms coexist (Boklund et al., 2020).

Because of their role as ASFV maintenance hosts, attempts to reduce wild boar populations and to manage ASF in wild boar are taking place, mostly in Europe and in South Korea. In the context of ongoing ASF expansion, the goal of this contribution was to learn from the successes and failures of the ongoing interventions for ASF management in wild boar populations and to propose improvements.

Eurasian wild boar and ASF

The wild boar is native to northern Africa and to Eurasia, from the Iberian Peninsula in the west to Japan in the east. Moreover, escaped or released domestic pigs, and sometimes pure or crossbred wild boar, have been introduced in many other regions, worldwide, and have established large and continuous populations in large parts of North and South America and Oceania. Invasive feral pigs cause crop damage and conflicts and represent a major threat to biodiversity (Alves da Rosa et al., 2017). Both native wild boar and feral pigs are precocious and prolific, adaptable, and extremely resilient to population control. Thus, wild boar (and feral pig) populations will grow 5-15% per year unless fecundity is lowered due to food scarcity, a huge proportion of the population (2/3) is harvested annually, or diseases contribute significantly to population regulation (Keuling et al., 2013, Massei et al., 2015, Barasona et al., 2016).

Both wild boar and feral pigs can potentially maintain ASF, although all recent evidence refers to the former, mostly in Europe, where spill over and spill back events occur from wild boar to domestic pigs and vice versa (Sauter-Louis et al., 2021). When ASF enters a naïve wild boar population, the expected events include (1) an 85–95% drop in total population density after the initial epidemic waves, (2) a peak in the number of infected individuals approximately 6 months after the virus is initially discovered, and (3) the persistence of the virus in the population for several years (O'Neill et al., 2020). This is observed both in Europe (EFSA AHAW panel et al., 2021) and in South Korea (Jo & Gortázar, 2021).

ASF control options in wild boar and feral pigs

The control of infections shared with wildlife, including ASF, starts with proper integrated wildlife monitoring (IWM), meaning setting up and maintaining a complete active and passive disease surveillance scheme combined with accurate population abundance monitoring (Cardoso et al., 2022). Provided such an IWM is running, control options are limited to three fields: biosafety and prevention, population control, and vaccination (Gortázar et al., 2015). In the specific case of ASF however, and despite recent progress, the latter option is not yet available (see other contributions in this issue). Furthermore, the possible interventions will depend on the epidemiological situation of the target region, where we could distinguish regions at risk, regions near ASF outbreaks or epidemic fronts, and already infected regions (Figure 1).

In regions at risk, such as all currently unaffected countries, prevention is the main goal and surveillance for early detection should be boosted. The available tools include preparing the farmer and hunter communities and the public

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for early ASF detection and delivering messages on farm biosafety, hunting and game meat hygiene, and ASF control to the appropriate stakeholders (Urner et al., 2021). Also, recreational hunting could be used to control or at least stabilize wild boar population numbers wherever possible. Alternatives such as habitat management by fencing off certain crops or targeted forest management would be desirable but are often long term and costly (Gortázar & Fernández-de-Simón, submitted). Finally, it is important to train the veterinary and environment or fish and game authorities to deal with an ASF emergency: the logistics involved just in carcass search and destruction are huge and need to be programmed timely. In front of an outbreak the first measure is assessing its geographical spread and its possible origin in time. Once ASF is detected in a wild boar population, European and Korean veterinary authorities generally follow established European recommendations for ASF control in wild boar (EFSA, 2014; 2018a). This includes a combination of three strategies: removing carcasses rapidly to avoid further infection transmission, confining ASF by fencing, and reducing wild boar density letting ASFV do its job and eventually by professional culling (Jo & Gortázar, 2020). In some countries, hunters perform important activities such as the removal of carcasses, fencing, or intensifying hunting (Urner et al., 2021). Immediate emergency interventions, however, generally stay away from recreational hunting to avoid further infection spread. In already endemic situations, prevention is no more an issue and recreational hunting and habitat management come again into consideration, alongwith carcass destruction and fencing.
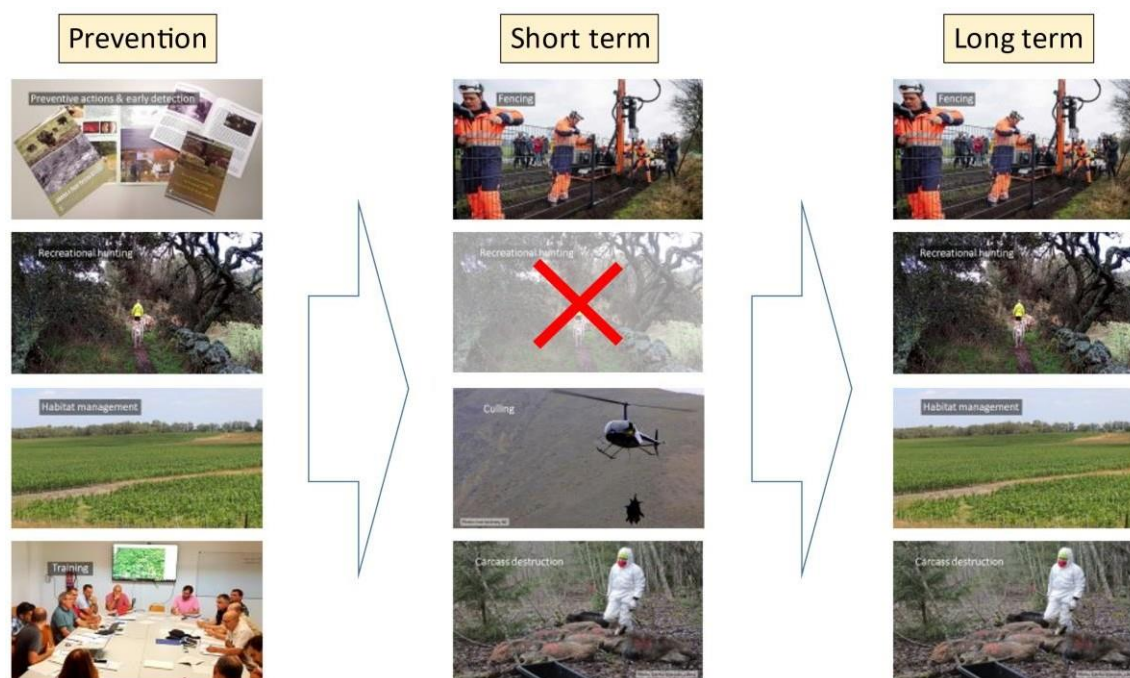
Figure 1. Intervention options for African swine fever control in wild boar depend on the epidemiological situation.

Regarding the pig farming side, efforts on farm biosafety are of utmost importance. Indeed, a recent report regarding outdoor pig farming in Europe indicates that the implementation of certain Biosecurity Measures (BSMs) and the assessment of on-farm biosecurity using standard protocols, would reduce the risk of ASF introduction and spread, while stating that quantitative information on the effectiveness of on-farm BSMs is lacking (EFSA AHAW panel et al., 2021). Also, backyard or small holdings with different biosafety capacities can spark outbreaks yet are often not addressed by standard farm biosafety policies (Smith & Dunipace, 2011). Little is known about how farmers practice biosecurity, except for certain production systems and geographical regions. For instance, innovative wildlife risk mitigation protocols are currently implemented locally in extensive pig farming in Spain (Jiménez-Ruiz et al., 2022). Finally, there remain significant gaps regarding farm biosafety assessment and monitoring.

Outcome of interventions

The current ASF panzootic coincides with far larger wild boar populations and notable habitat and societal changes (Jori et al., 2021). This context might contribute to explain why, once ASF becomes endemic in a certain region, it is likely to persist for a long time, especially where habitat is favorable and original wild boar densities were high. Unfortunately, this is consistently observed despite deploying control interventions (Desmecht et al., 2021).

The prospects are slightly better for recent ASFV introductions. Interventions for ASF control in wild boar in outbreak and epidemic front situations were carried out or are still taking place in several European countries and in South Korea. This provides an opportunity to evaluate the outcome of these interventions.

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Table 1. Currently available field evidence on interventions for ASF control in infected wild boar populations

References	Region	Type	Interventions*	Outcome
EFSA et al. (2020)	Czechia	Local outbreak, early detected	In the infected zone, initiallyS & F, later S, F, C. In surrounding counties, C	Success
Dellicour et al. (2020)	Belgium	Local outbreak, detected after several months	In the infected zone, S, F, C. In surrounding counties, C	Success
Wozniakowskiet al. (2021)	Poland	Epidemic front	In the infected zone, S, F, C. In surrounding counties, C	Failure
Jo & Gortázar (2021)	South Korea	Epidemic front	In infected zones, initially, silent professional C, F and S (restricted due to mined zones); later, amateur C andS, leading to ASF spread. In surrounding counties, F	Failure
Sauter-Louis et al. (2020)	Germany	Epidemic front	S and F (restricted due to large area, wetlands, and legal issues), C in surrounding counties	Failure
E. Ferroglio (pers. comm.)	Italy	Large outbreak, detected late	Still in phase of zoning and intervention planning**	Uncertain

* C = wild boar culling; F = fencing; S = carcass search and destruction. ** As of 1 March 2022.

Table 1 lists the currently available field evidence on interventions for ASF control in infected wild boar populations. According to the available data, the likelihood of a successful ASF control in wild boar depends on the scale of the epidemic and the time interval between onset and first detection. Successful eradication was only achieved in two relatively small local outbreaks (Dellicour et al., 2020). Failures in large-scale epidemic front situations are first an obvious consequence of repeated incursions over large areas (Sauter-Louis et al., 2021) and may partially be explained by limitations to effective intervention deployment, e.g., incomplete fencing at the German-Polish border due to habitat characteristics (large wetlands) and legal issues, or incomplete carcass search and destruction in South Korea due to mined areas and access restrictions (Jo & Gortázar, 2021). Regarding local outbreaks, success is more likely but probably depends on early detection and intervention, as evidenced by the fact that the Czech outbreak lasted for one year after a very early detection, as compared to the longer period needed to control the Belgian outbreak. Interventions in Poland and in South Korea were possibly also hindered by the -mostly habitat mediated- high local wild boar densities (Wozniakowski et al., 2021).

These observations are in agreement with modelling results suggesting that, when only a single intervention is deployed, successful ASF control in wild boar is less likely, that ASF control likelihood improves if the time between the first case and its detection and subsequent intervention is short, and that high wild boar density at the start of the epidemic might hinder ASF control. Furthermore, other factors modulating ASF control success according to the model include wild boar feeding or habitat quality, and the presence or absence of high temperatures and obligate scavengers which might contribute to carcass destruction (Zani et al., 2020). These potential modulators deserve attention and field studies.

It is also pertinent to stress that long- and medium-distance spread of ASF (>30 km) is rather unlikely to occur due to wild boar dispersal (Taylor et al., 2021), and human-mediated dispersal is more likely. This implies that, even if all wild boar targeted interventions at the epidemic front are carried out consistently and efficiently, the human factor can still intervene and jeopardize the huge effort involved in infection spread containment.

Knowledge gaps and future perspectives

Knowledge gaps have been addressed recently by EFSA, prioritizing among others the following gaps: (1) role and efficacy of recreational hunting and professional culling for wild boar population control; (2) implementation of practical methods to estimate wild boar density; (3) holistic assessment of the factors that determine the presence of

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wild boar near to different pig farm types, including outdoor farms and extensive production systems; (4) acceptance of measures for wild boar management by hunters; (5) assess how to improve coordinated national and international decision-making; (6) basic aspects of wild boar population dynamics all over Europe; (7) the efficacy of different fencing methods with GPS-collared wild boar, considering also the effect on non-target species; (8) biosecurity awareness and implementation among backyard pig farmers; (9) efficacy of wild boar trapping methods including welfare implications and social acceptability; (10) effect of food availability in natural areas in relation to baiting and feeding on wild boar population dynamics; and (11) use of trained dogs in ASF-affected areas to manage wild boar populations. For each of these gaps, a research protocol has been proposed and, in several cases, applied research activities are already ongoing or are incentivized. Additional fields deserving research include the potential role of high temperatures and obligate scavengers in ASF control through their effect on carcass survival.

One specific aspect garnering attention is oral vaccination of wild boar against ASF and vaccine deployment through baits. As this strategy of vaccination and drug delivery has been successful in combating other infections shared with wildlife, there is a need for the development of vaccines and of stable and specific baits, as well as the development of better delivery systems for such baits. A definitive effective vaccine has not been developed yet, although there is progress with different vaccine candidates (Bosch-Camós et al., 2020; see also other contributions in this issue). The ideal bait would be cheap and easy to produce, stable against water and extreme temperatures, hard enough to allow aerial deployment, species-selective and suitable for all age-classes.

Figure 2. Baits developed at IREC institute to deliver vaccines to wild boar.

Conclusions

Four years ago, a large group of wild boar experts argued that disease control interventions with a major impact on wildlife management and conservation, such as large-scale fencing and culling, should consider implications beyond animal health. They urged governments to agree on a coordinated and cross-departmental response that adheres to the principles of modern adaptive wildlife management, also considering the human dimension and improving wildlife population monitoring (Vicente et al., 2019). Although significant milestones have been reached, particularly regarding wildlife population monitoring in Europe (www.enetwild.com), there remain significant needs regarding improved and science-based wild boar population control and regarding farm biosafety, among many others.

Wild boar and feral pig populations are globally on the rise, mostly due to habitat favorability. Thus, ASF might be expected to continue to spill over into wild suid populations. Controlling ASF, like in the case of other shared infections where vaccines are currently not available, is limited to biosafety and prevention (including fencing and carcass destruction) and to population control. Furthermore, the available intervention options depend on the epidemiological situation of the target region while the success likelihood of these interventions will also depend on the geographical and time scales of the event. So far, only two success stories of ASF control are known, and both refer to local outbreaks. Unfortunately, there is no precedent of successfully halting ASF spread in an epidemic front. Abundant research is ongoing and hopefully the future will see vaccines as one more tool in the box.

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A current global view of the asf situation

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Introduction

The current global situation of African swine fever (ASF) is currently worse than never since 1921 when was firstly described by Montgomery in Africa. The disease is affecting to the five continents: Africa, Europe, Asia, Oceania and America (Dominican Republic and Haiti).

Today, ASF is the biggest threat of the global swine industry

The ASF virus has left Africa three occasions since 1921. The first in 1957 to Portugal: The leftover from a plain from Africa was given to the pigs of a small farm near the Lisbon airport. In this occasion the virus isolate was from genotype I and not high virulence. The Portuguese was working on it control, but similar situation happen again in 1960, but in this occasion¸ a very high virulence virus (Lisbon 60), also from genotype I, that spread very fast to Spain 1961, France 1967, Malta, 1978, Belgium, 1985 and Nederland 1986, Cuba 1971 and 1980, Brazil 1978, Dominican Republic 1978 and Haiti 1979. (OIE WAHIS) and (Sanchez-Vizcaino et al., 2019).

The third visit of the ASF virus, has been until now, the worse visit of the ASF. A high virulence virus genotype II left the east coast of Africa by boat in April 2007 to the harbor of Poty in Georgia, where again, the leftover of the boat was given to a group of pigs that become infected of ASF. From there, the virus spread to Armenia, August 2007, Russia, Nov 2007 in wild boar and June 2008 in domestic and the disease continuing spread to Europe, EU in 2014, Asia in 2018, America in 2022. Five continents more than 50 countries in the worse scenario never observer before. (OIE WAHIS) and (Sanchez-Vizcaino, et al., 2019).

Two important conclusions were observed in this last visit of the virus: one, the food from infected pigs is still the most important mechanism of transmission of the ASF virus to long and short distance. Second, the infected wild boar was the second important factor for transmission to short and medium distance in an infected country.

In this presentation, a global view of the current ASF will be review, from Africa, Europe, Asia to the American continent, given a special attention to the ASIA continent, where 17 Asian countries are affected (February 5, 2022) and both virulence and attenuated ASF genotypes I and genotypes II are circulating (Chen Weiye et al., 2020), (Encheng Sun et al., 2020 and 2021) as well as illegal vaccine from both genotypes. The current main factors related with these epidemiological scenarios will be review as well as the epidemiological implication will be evaluated.

Finally, an update information relates with the development of the EU VACDIVA (https://vacdiva.eu/): An ASF SAFE, DIVA and EFFECTIVE vaccine for domestic pigs and wild boar will be presented.

Situation of ASF in Africa

The ASF virus was first described in Kenya by Montgomery in 1921, after an introduction of pig from England that died with a very acute haemorrhage disease different than classical swine fever.

In Africa the current situation of ASF could be divided in two different epidemiological scenarios, both of them endemic to the disease. The west part where the disease is mainly presented in domestic animals with outbreaks in backyard and family small farm and the easter part where the 24 ASF genotypes described until now are present and circulating and the sylvatic and domestic cycle coexist. Vectors and reservoir of the virus: wild pigs (Phacoccero and Bush pigs) and vectors ticks, the Ornithodoros ssp (*O. mubata*, *O. porcinus domesticus* and *O. porcinus porcinus*)and domestic pigs.

This is the most complicate scenario because the wildlife act as chronic carriers (look like tolerance) with very low virus (10to2) but the *O. Mubata* can amplify the virus from 10to2 to10to7 and infected domestic pigs and wild live.

Here the control and eradication is almost impossible for the protection to the wild life until a safe and effective vaccine could broke this sylvatic cycle.

Situation of ASF in Europe

In Europe the ASF virus enter in the harbor of Poty, Georgia in 2007 and since them 20 different countries where infected until now: Georgia, Azerbaijan, 2007, Russia, in 2012, Ukraine, and Belarus 2013, Lithuania, Poland, Estonia and Latvia in 2014, Moldova 2016, Rumania, 2017, Check Republic 2017, Bulgaria, Hungary and Belgium 2018, Slovakia and Serbia 2019, Greece and Germany, 2020, and the last one until (March 04,2022) Italy 2022. (OIEdifferent report information). From these 20 countries affected only in the Check Republic and Belgium, the disease was eradicated. The ASF infection in those countries was affecting only to wild boar (OIE WAHIS) (de la Torre, et al., 2022).

In Europe two different epidemiological scenarios are observed. In the East part, where most outbreaks are reported in domestic pigs following the wild boar and the West part where most cases are reported in wild boar: 64% in the natural forest area, 20% in the agroforestry area and 12,4% in the Agro urban area, with some sporadic outbreaks in domestic. This distribution is related with the low biosecurity of the farms and its localization, some of them, in or near, the forest areas (Bosch et al., 2020).

Situation of ASF in ASIA

In Asia continent currently 17 of 28 countries are infected of ASF (OIE WAHIS, FAO EMPRES).

In 2018 the virus was first described in China, since there 17 more countries became infected. Both ASF high virulence isolates, genotype II, and attenuated genotype I, are circulating in China related with the initial infection (genotypes II) and the illegal vaccine produced in China genotypes II and I. (Encheng Sun 2020 and 2021).

This situation created important epidemiological implication to all ASIA and the rest of the world, due to the big amount of contaminated pig meat and pig products circulating. These virulent and attenuated ASF isolates, in particular these attenuated ASF isolate, could create great difficulties for the early detection of ASF around the world because the clinical presentation and lesions are very different than the high virulence isolate. The attenuated clinical isolate induces a longer incubation period and chronic forms characterized by inflammation of the joints, generally more frequent in hind legs and skin lesions.

The specific situation of each ASIA country will be also review. Up today look like only the high virulence genotypes II isolates are circulating in the other 16 infected countries. Special attention should be dedicated in the future to this important risk point.

Other risk factor detected was the potential implication of wild boar and the transmission of the disease in the area. In general, the high population of wild board in the area is not correlated with the number of cases declared except in South Korea. In China only 9 cases of wild boar have been reported. This is an important factor because in general the wild boar play an important role in the transmission of ASF. (Bosch et al., 2017), and (Cadenas-Fernandez (2019).

Other important risk to keep in main, as was already described, is the possible problems of attenuated ASF isolates circulating out of China. (Encheng Sum 2020 and 2021) and (Weije Chen et al. 2020). This could complicate very much the early detection of new ASF introduction. More information to the veterinarian and farmers should be done in order to be familiar with the lesion of this attenuated isolates.

In relation with the situation of ASF in China is important to keep in mind that China has increase its engagement with Latin America and the Caribbean Chine (LAC) has signed various bilateral partners sheep with several countries of the area such: Argentina, Brazil, Chile, Ecuador, Mexico, Peru and Venezuela.

In relation with outbreaks information and data availability is very difficult to track the new outbreaks in specific time period. The OIE and FAO are doing a great job but some time, even for those important sources, the informationdoes not match, but still is the best source to following the situation in China.

How about a vaccine for ASF?

There have been many attempts to obtain a vaccine for ASF since 1921without irregular success.

In the 1960s, when the disease left the African continent for the first time and infected Portugal and later Spain and other European countries, the idea of obtaining a vaccine has been on the minds of researchers. In the 1960s, first in Portugal by Dr. Manso Ribeiro and his team, and later also in Spain by Prof. Sanchez Botija and his team, attenuated

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vaccines were developed by different passages of the ASF virus and pigs leukocytes that demonstrated protection against circulating viral strains but also side effects and development of chronic forms of the disease affecting the pigs skin and producing joint lesions, mainly of the hind legs. The balance between effectiveness and safety did not seem easy to achieve. Since then and up to date, several strategies have been used, obtaining the most promising results the vaccine prototypes with attenuation either generated naturally, by natural infection in different animals or by successive passages of the virus in porcine leukocytes or in adaptation to other cells. The inactivated vaccines, of subunits with different proteins, the DNA vaccines, have never generated significant protection. (Muñoz-Perez et al, 2021).

The enormous complexity of the virus with more than 170 genes, most of them still not well known, the lack of neutralizing antibodies, at least in significant quantity, made it difficult to obtain a good balance between protection and safety. However, advances in the molecular biology of the virus and the knowledge of some genes related to virulence have allowed significant advances and great hope for the production of safe vaccines (Muñoz-Perez et al, 2021).

Several research groups are working in Europe, USA and Asia in the development of a vaccine against ASF, with promising results. One of these projects is the **EU VACDIVA project. (https://vacdiva.eu/):**

The EU VACDIVA project: A safe, DIVA and effective vaccine for domestic pigs and wild boar

The VACDIVA consortium started in October 2019 and will be finished in October 2023. Is integrated by thirteen research laboratory and epidemiologists' partners from the EU with high experience in ASF and wild life and two important companies: MSD and Ingenasa, plus three partners from outside of the EU: China, Russia and Kenya.

The objectives of the EU VACDIVA project are:
1. To obtain a safe, DIVA and effective vaccine for domestic pigs (by inoculation) and wild boar (oral vaccine).
2.   A DIVA diagnosis test that can distinguish animal vaccinated of infected animal by PCR and ELISA or other serological tests.
3. To design an ASF control and eradication strategy for different epidemiological scenarios worldwide and test the pilot vaccine in real controlled environment.

The VACDIVA project vaccine candidates are based in natural attenuated ASF isolate, from genotype I and II, obtained from wild boar or domestic pigs that induced important cross protection against different ASF high virulence viral isolates. As well as mutants obtained from these attenuated isolates by different type of gene deletion that included complementary safety and good DIVA market to the candidates.

At this moment, we are continuing evaluating the different vaccine prototypes in domestic pigs and wild boar and preforming several in vivo experiments to assure **the safety, the DIVA test and the efficacy of the vaccine.** The results obtained until now proved an efficacy against heterologous high virulence isolate between 92 to 100% protection.

To have **a safe, DIVA and effective vaccine** is the main goal of the EU VACDIVA project. The results obtained until now are very promising, but more studies on immunity duration, security in pregnant, young animals and cross protection should be finished. (hllps://vacdiva.eu).

Some final conclusions and comments:

1. The global situation of ASF is worst that never since the first description of the disease in Kenya in 1921 by Montgomery.
2. The ASF is continuously spread from 2007 with no stop and affecting already the five continents and more than 50 countries. Only in two countries (Check Republic and Belgium) the disease has been eradicated so far.
3. The main mechanism for long distance transmission is the swill feeding from ASF contaminants meat and products, normally starting in backyard, family farm and wild boar.
4. Other mechanisms of transmission for short and intermediate distance are the infection of the wild boar and combination with low biosecurity farms, swill feeding and vehicles, movement of people and insufficient compensation to farmers, illegal trade,
5. A good surveillance program, good biosecurity and early detection, are the best and effective tools to prevent the ASF introduction in a country or free area.
6. Information to clinical veterinarian and farmers about the circulations of virulence and attenuated ASF virus isolation is critical for an early detection of ASF.

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7. The surveillance program should be continually adapted to the current's risks. The introduction of contaminates pigs' meat or pig products is today the most important global risk to prevent the first introduction.
8. Special attention of the surveillance program should be dedicated to the control of garbage from harbors (especially commercial containers boat), small private airport, tourisms, border, etc.
9. Do to the large amount of contaminated pig meat and pigs products circulating the whole planet is at riskof ASF infection.
10. One ASF vaccine is closer than never but still not in the market.

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Development of recombinant live attenuated vaccine candidates in ASF

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African swine fever (ASF) is a devastating disease of swine present in more than twenty sub-Saharan African countries and currently being considered a pandemic affecting countries in a large contiguous geographical area from Central Europe to East and Southeast Asia. In the last months, the disease was also detected in Dominican Republic and Haiti, after more than fifty years of being absent from the Western hemisphere. This situation is causing significant economic losses in the pig industry and a shortage in food availability in the affected countries. The causative agent of this epidemiological situation, African swine fever virus (ASFV) strain Georgia 2007 (ASFV-G), is a highly virulent strain that belongs to the ASFV genotype II group.

ASFV is a large and structurally complex virus, presenting a double envelope and harboring a large (180-190 kilobase pairs) double-stranded DNA genome, encoding approximately 150-160 genes. The role of most of these genes remain unknown or just have been predicted by functional genomics. Actually, just a few virus proteins have been experimentally studied to determine their function.

Currently, there is no commercial vaccines available to prevent ASF, so control of the disease is basically achieved by culling the affected animals. The current ASF pandemic has energized the research in the development of experimental vaccines to improve the epidemiological management of the disease. Among the experimental vaccines, those based in the use of attenuated strains constitutes the most successful candidates. Although approaches to develop attenuated ASFV strains have considered the use of naturally attenuated isolates as well as those obtained by adaptation to grow in cell cultures, vaccine candidates rationally designed and developed by genetic manipulation are the very promising alternatives. Attenuation of virulent viruses have been achieved by deleting ASFV genes associated with virulence in swine through genetic manipulation. Using this approach several experimental recombinant vaccine candidates have been developed which efficiently protect pigs against the challenge with the ASFV-G isolate or its field isolate derivatives. Therefore, discover of virus genes important in the process of disease production is a critical first step in the design of recombinant LAVs to prevent ASFV infection and disease. Discovering ASFV genes involved in virus virulence is a laborious and time demanding process based, essentially, on a systematic experimental approach. At the moment, only fifteen ASFV genes or a group of genes, have been identified as determinant of virulence since their individual deletion produced a significant decreased of virulence of the corresponding parental virulent strain. Nine of these determinants of virulence have been shown to produce attenuation in the ASFV-G, or any of its derivatives, and those have been demonstrated that can be used to develop an attenuated strain able to induce protection against the challenge with the field isolate responsible for the current pandemic affecting Eurasia. The deletion of even highly conserved virus genes have been shown to have different consequences in terms of virus virulence depending on the virus strain considered. These factors make the identification and characterization of novel genetic determinants of virulence an essential issue for the rational production of the next generation live attenuated vaccine candidates to protect swine against the current pandemic strain ASFV.

Here, we will review the different attempts performed in our laboratory in the development of recombinant live attenuated vaccine candidates inducing protection against the Georgia isolate. The design and development of these recombinant viruses will be analyzed and discussed in some detail.

Our first attempt to develop a live attenuated strain based in the ASFV-G isolate was the deletion of the 9GL gene (O'Donnell et al., 2015b). Deletion of 9GL gene produced attenuation of the ASFV-G virulent phenotype when used at relatively low doses (less than 10^3 HAD50) but showed residual virulence a higher dose. Regardless its residual virulence, when used at sublethal dose (at 10^3 HAD50 or less), ASFV-G- 9GL induced an efficacious protection against the IM challenge with the ASFV-G isolate both, at 21 and 28 days pv. ASFV-G- 9GL was one of the first recombinant attenuated viruses reported to induce protection against the epidemiologically important Georgia isolate.

To increase safety profile to ASFV-G- 9GL we combined the deletion of the 9GL gene with the additional deletion of the UK gene. A virus harboring deletions of both genes, named ASFV-G- 9GL/ UK, presented a drastically more attenuated phenotype than the parental ASFV-G- 9GL (O'Donnell et al., 2017). Animals inoculated with up to 10^6 HAD50 of ASFV-G- 9GL remained clinically normal and were effectively protected against the IM challenge with 10^3 HAD50 of Georgia isolate.

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Attenuation of virulent ASFV was also achieved by deleting 6 genes belonging to the MGF360 and MGF505 groups from the genome of the highly virulent ASFV-G isolate (O'Donnell et al., 2015). Pigs inoculated IM with up to 10^4 HAD50 of the resulting recombinant virus, ASFV-G- MGF, remained healthy, without signs of the disease and, when they were challenged with virulent parental ASFV-G strain, no signs of the disease were observed although a proportion of these animals harbored the challenge virus.

Another single gene deletion recombinant virus with potential vaccine capability was developed by deleting the I177L gene from the genome of the virulent ASFV-G (Borca et al., 2020). The recombinant virus lacking this gene, ASFV-G- I177L, present a drastic decrease in virulence when inoculated in pigs. Even those receiving up to 10^6 HAD50 IM remained clinically normal and were completely protected against the challenge IM with ASFV-G virus at 28 days pv, even those receiving as little as 10^2 HAD50 of ASFV-G- I177L. Animals vaccinated with doses of 10^4 HAD50 or higher of ASFV-G- I177L developed sterile immunity against the challenge virus.

Interestingly, efficacy of ASFV-G- I177L was also tested using as challenge virus a highly virulent field isolate from Vietnam, TTKN/ASFV/DN/2019 (Hanh et al., 2021). ASFV-G- I177L induce protection against TTKN/ASFV/DN/2019 challenge with a similar efficacy than against the Georgia2007 strain in experiments conducted in parallel using pigs with both, European (Yorkshire/Landrace crossbread) as well as Vietnamese genetic background (Mong Cai breed).

ASFV-G- I177L was also tested when administered by oronasal route (Borca et al., 2021), exploring its potential use as an oral vaccine. Animals oronasally inoculated with ASFV-G- I177L and IM challenged 28 days later with the virulent ASFV-G isolate were protected, not showing clinical signs associated with ASF.

A modification of ASFV-G- I177L has been obtained by adapting the virus to grow in an established swine cell line, as a mean to facilitate its production at industrial scale (Borca et a., 2021b). The adapted virus, ASFV-G-I177L/ LVR, showed a large and stable deletion in the left variable region of its genome. Challenge studies performed in domestic pigs showed that ASFV-G- I177L/ LVR maintained the same level of attenuation, immunogenic characteristics, and protective efficacy as ASFV-G- I177L.

Finally, deletion of the ASFV gene A137R has also shown to attenuate ASFV-G isolate (Gladue et al., 2021). Removal of the gene from the genome of the highly virulent parental virus produced a virus, named ASFV-G- A137R which, when IM inoculated in pigs (10^2 HAD50) showed a strong attenuation. Interestingly, all ASFV-G- A137R inoculated animals were protected when IM challenged with the virulent parental strain ASFV-G without showing evidence of replication of challenge virus.

An update of the status of development of these vaccine candidate strains will be presented along with several potential recombinant vaccine strains developed in other laboratories.

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The global human and swine influenza A virus interface

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Introduction

H1N1, H1N2, and H3N2 influenza A virus (IAV) subtypes are endemic in swine herds around the world. Swine H1 genes are classified into three major genetic lineages: 1A, 1B, and 1C (Anderson et al. 2016). The 1A lineage arose from the 1918 human pandemic H1N1, with global introductions into swine and subsequent transmission and migration. The 1B lineage resulted from repeated independent global introductions of human seasonal H1 IAVs into swine. The 1C lineage arose from an avian IAV spillover into swine. Swine H3 viruses also arose from multiple independent introductions from human seasonal H3N2. H3 genes in swine are classified by the decade of introduction, 1970s, 1990s, and 2010s (Anderson et al. 2020). Since 2009, frequent human to swine transmission of H1N1pdm09 (clade 1A.3.3.2) IAV occurred and viral diversity in all swine IAV lineages increased after reassortment with co-circulating H1N1pdm virus internal genes (Nelson et al. 2015).

Because of the risk animal IAV pose to the human population, experts at the World Health Organization (WHO) vaccine composition meeting review zoonotic cases and consider them for development of pandemic-preparedness candidate vaccine viruses (CVV). Few of the genetic clades globally detected in swine currently contain a CVV and those available may not provide protection given observed genetic and antigenic differences in circulating swine viruses. Human and swine IAV evolution are entwined, particularly since 2009, therefore, a system to prioritize and evaluate evolving swine IAV in the context of human risk should be part of a comprehensive pandemic preparedness plan. A more systematic analysis of swine IAV as a risk to the human population is a priority of the OIE and FAO influenza network (OFFLU). The objective of this study was to characterize the genetic and antigenic diversity of swine H1 and H3 with contemporary global detections to inform pandemic preparedness for human public health.

Materials and Methods

We conducted analyses of swine and human HA sequences by subtype and lineage or clade. Reference sequences and new data deposited July 1 to December 31, 2021, were downloaded from GISAID or GenBank and aligned with MAFFT (Katoh & Standley, 2013) using default settings. Alignments for each segment were inspected manually and trimmed to the start and stop codon. Phylogenetic trees were generated using FastTree (Price et al., 2010). Tabular comparisons between current CVVs or human seasonal vaccine strains and new swine data were generated using the NADC IAV bioinformatic toolkit (https://github.com/flu-crew). An HA1 consensus sequence was generated for each contemporary clade and the best matched strain was selected for testing against reference ferret antisera in hemagglutination inhibition (HI) assays. All HI assays were performed with ferret anti-sera and guinea pig red blood cells. H3N2 assays were performed with the addition of oseltamivir.

Results

Phylogenetic analysis identified HA sequences in nineteen genetic clades from H1 and H3 IAV collected from swine between July 1 to December 31, 2021 (Figure 1). Twelve clades were H1 subtype from each of the lineages: 1A classical swine lineage (5 clades); 1B human-seasonal lineage (4 clades); and 1C Eurasian avian lineage (3 clades). The 1A classical swine lineage viruses have global detection: 1A.1.1 in USA and Canada; 1A.2 in Canada; 1A.4 in USA; 1A.3.3.3 in USA. The 1A.3.3.2/pdm circulated in all countries that collected sequence data. 1B.1 human seasonal lineage was only in Europe, and the 1B.2 human seasonal lineage was only in the USA. The 1C Eurasian avian lineage was reported in Europe. Seven clades were within five distinct lineages derived from human seasonal H3 virus spillovers grouped by the decade of introduction into swine (1970.1; 1990.1; 1990.4; 2010.1; 2010.2). The H3 1990.1, 2010.1, and 2010.2 clades were only detected in the USA and the H3 1970.1 clade in Europe. The 1990.4 lineage circulates in the USA, Canada, and Mexico.

During the data collection period, 15 human zoonotic cases of swine IAV origin (termed variants) were detected in 5 countries: USA: H1N1v (3 1A.3.3.2/pdm, 2 1A.3.3.3), H1N2v (3 1B.2.1), H1v (no sequence), H3N2v (1 2010.1; 1

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1990.4a), Canada: H1N2v (no sequence); Denmark: H1N1v (1A.3.3.2); France: H1N2v (1C.2.4); Austria: H1N2v (1C.2.4). An additional 6 variant cases were reported but collected outside the report window in 3 countries: Taiwan: H1N2v (1A.1.4); China: H1N1v (4 1C.2.3); Australia: H3N2v (Other-Human-1990).

Antigenic analysis showed variable, but overall limited recognition of contemporary swine IAV by antisera against pre-pandemic CVV or seasonal human vaccine strains. In the USA, Europe and the UK, 1A 3.3.2/pdm swine IAV showed variable reactivity vaccine antisera. 1B 1.2.3 viruses representing a significant proportion of viruses currently detected in Europe showed no cross-reactivity to reference sera. 1C: 1C2.4 and 1C2.5 viruses represent a genetically diverse and significant proportion of 1C lineage viruses detected in Europe and showed inconsistent cross-reactivity to vaccine anti-sera. H3 1970.1 lineage viruses demonstrated cross-reactivity with older human seasonal anti-sera.

In the USA, 1A.1.1 viruses demonstrated a significant loss of cross-reactivity against the within clade CVV and represent a consistent proportion of 1A lineage viruses detected in U.S. pigs. 1A.3.3.2 swine strains exhibited a loss in cross-reactivity with current human seasonal vaccine strains. 1A.3.3.3 clade-3 is a substantial proportion of 1A lineage viruses detected in U.S. pigs and a representative strain demonstrated a 16-fold decrease against the tested 1A.3.3.3-clade 1 CVV. A variant case within the 1A.3.3.3-clade 3 was recently recommended for development as a CVV but anti-sera was not available for testing. An additional two variant cases were reported from 1A.3.3.3-clade 3. 1B.2.1 cross-reacts with the within CVV. This clade contained three reported H1N2v during this reporting period. 1B.2.2.1 ranged from 2- to 4-fold reduction from the within clade CVV. There is no within clade CVV for 1B.2.2.2 and the representative strain demonstrated a significant loss in cross-reactivity against the 1B.2.2.1 CVV. 1B.2.2.1 and 1B.2.2.2 clades represent a low but consistent proportion of 1B lineage viruses detected in U.S. pigs. A representative H3.1990.4.a lineage virus demonstrated a significant loss of cross-reactivity against the within clade CVV and represent a substantial proportion of H3 viruses detected in U.S. pigs. H3.1990.4i lineage viruses demonstrated significant loss of cross-reactivity against all CVV and human seasonal vaccine strains. These swine viruses are less frequent but consistently detected. H3 2010.1 and 2010.2 lineage viruses had significant loss in cross-reactivity with both the 2010.1 CVV and to the human vaccine strains. H3 2010.1 lineage viruses are a substantial proportion of H3 viruses detected in U.S. pigs.

Conclusions and Discussion

IAV in swine is highly diverse, with sustained transmission in global pig populations of at least 30 HA genetic clades detected in recent years and 19 in the 6-month window of this study. The NA and other 6 gene segments of swine IAV also demonstrate a high degree of diversity. Although most of the current swine lineages arose from interspecies transmission of human seasonal IAV into swine, contemporary swine IAV were significantly different at the genetic level from the current H1 and H3 components of human seasonal IAV vaccines. Although 1A.3.3.2/pdm continue to be transmitted from human to swine, maintenance and onward transmission in swine was associated with genetic diversity in the HA gene and variable cross-reactivity at the antigenic level with human seasonal vaccine anti-sera. Many of the distinct genetic clades of HA genes detected in swine globally do not currently contain a CVV or human seasonal vaccine, and the degree to which those CVVs provide protection is uncertain given observed genetic and antigenic differences identified in recently circulating swine viruses.

Since human and swine IAV evolution are inherently tangled, a system to prioritize and evaluate evolving swine IAV in the context of human risk should be part of a comprehensive pandemic preparedness plan. During the time period of this study, HA genes from swine IAV were reported from only 5 countries, with sporadic and inconsistent levels of data over the recent past. Surveillance in swine must continue to be a priority for animal and public health, with priority given to geographic areas with high levels of swine IAV diversity, rapid evolution, production practices that support viral transmission and migration, as well as specific animal-human interfaces that promote greater contact between pigs and people.

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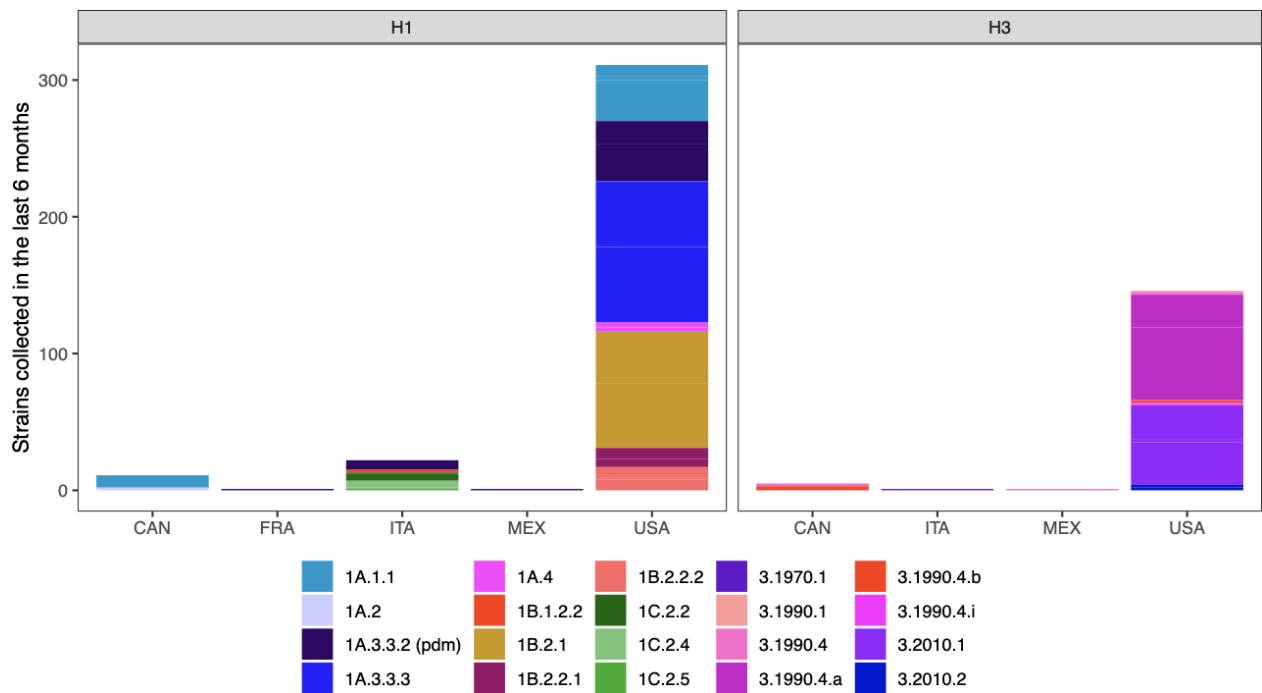
Figure 1. Geographic Distribution of swine hemagglutinin gene phylogenetic clades by country. Summary of swine hemagglutinin (HA) genes by country are colored by phylogenetic clade for sequences collected July 1 to December 31, 2021 (n=499).

African swine fever: Prospects for using knowledge of the virus to improve control of this global threat throughout diagnostic point of view

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Summary

African swine fever virus (ASFV) is a complex DNA virus that has a significant impact on the global swine industry. The lack of a safe and effective vaccine and the reliance on herd culling to prevent the spread of the disease has resulted in significant economic losses. Therefore, improved early detection remains a significant priority. Despite the great effort made in the last five years, there are still some gaps to fill. The low rate of variation of the ASFV genome and its enormous size make it difficult to properly type newly emerging ASFV isolates and thus make it difficult to trace outbreaks. An international effort should be made to develop a standardized genotyping method based on multiple loci of the ASFV genome to identify the origin of outbreaks. The trend towards endemicity of ASFV in the affected regions of Europe and Asia increases the presence of less virulent strains that induce non-specific clinical signs and make it difficult to recognize the disease in the field. It is necessary to increase knowledge about the mechanisms of spread and persistence of ASF in endemic areas and elucidate the role of animals that survive the disease, that is, the role of seropositive animals as potential carriers based on diagnostic data. These data are essential to determine the dynamics of the infection in the affected countries and support control and eradication programs. The use of alternative samples, such as blood or oropharyngeal swabs, may support these programs. While these alternative approaches are feasible, they should be integrated into control strategies by selecting test methods and sample materials following a "fit for purpose" approach. Finally, since there is a strong demand for the development of accurate, fast and simple detection methods, especially for their application in situ, important efforts must be made to validate them in the field at an international level.

Current circulating asfv strains

Genetic characteristics

The investigation of virus molecular evolution in combination with spatio-temporal data is an integral part of pathogen tracing and may help in the identification of potential routes of its spreading, therefore in disease prevention and control. The genome of the African swine fever virus (ASFV) is very complex with approximately 170 to 190 kilobase pair and more than 150 genes. The differences in size between the different strains are due to insertions or deletions at the ends of the genome where the multigene families are located. Variations in the conserved central region related to single nucleotide polymorphism (SNP) or to the presence of tandem repeat sequences (TRS) have been also described. ASFV isolates are classifying in 24 genotypes (figure 1) by the comparative analysis of the C-terminal end of the *B646L* gene, which encodes the p72 protein (Quembo *et* al.,, 2019). This method, used internationally, allows relatively quick and easy typing of ASFV strains and remains the first approach to identify the origin of the virus in case of introduction into new territories. The 24 circulating ASFV genotypes are present in Africa, with genotype I being the predominant one in West African countries where the sylvatic cycle is minority. Outside of Africa, genotype I was linked to the old historical ASFVs that were circulating in Europe and America until the mid-1990s. This genotype has remained endemic only in Sardinia (Danzetta *et* al., 2020).

In 2007, genotype II jumped from East Africa to the Republic of Georgia in Europe (Rowlands *et* al., 2008). Subsequently, this genotype spread throughout the Caucasus region, the Russian Federation and Eastern Europe, reaching the European Union (EU) in 2014 (Gallardo *et* al., 2014). Since then, 13 EU countries have reported ASF cases in the EU, mostly in wild boar. The most recently affected EU country was Italy, with genotype II detected in dead wild boars in the Piedmont region of north-western Italy. Two European countries have managed to eradicate the disease: Belgium (event resolved in March 2020) and the Czech Republic (event resolved in April 2018). In August 2018, China reported the first ASF outbreak in Liaoning province caused by a genotype II strain (Ge *et* al., 2018). By the end of February 2022, ASFV had been detected in 32 provinces in China and 13 Asian countries, the last being Thailand in January 2022. In September 2019, the first occurrence of ASFV was detected in Oceania, in Timor-Leste, followed by Papua New Guinea (March 2020). In July 2021, the disease reappeared in the Americas after an absence of almost 40 years, with new outbreaks detected in the Dominican Republic and later in Haiti (FAO Situation Update, www.fao.org).
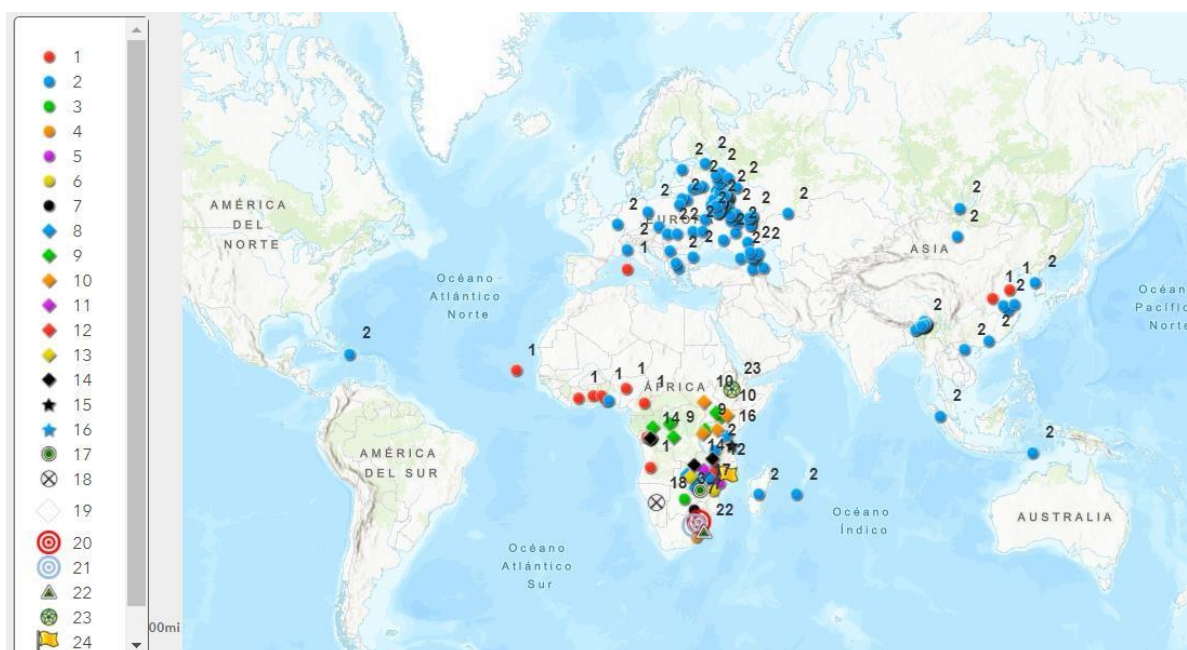
Figure 1: Distribution of the ASFV genotypes (updated March 2022).

The ASFV genome is a very stable virus with low genetic variability. Currently there are 139 complete genomic sequences of 13 of the 24 genotypes described, and 41 correspond to genotype II. The mutation rate among genotype II isolates is less than 0.3% (Table 1). Therefore, complete sequencing of the virus genome is not a viable method for rapid genotyping to identify the source of an outbreak.

Table 1: Percentage of identity amongst 41 ASFV genotype II isolates (39 from Europe and Asia and 2 from Africa) based on the comparative analysis of the whole genome sequences available at Genbank (cover >90%)

ASFV	Country	Year	Percentage of identity	Cover
Georgia2007/1	Georgia (index case)	2007	100,00%	100%
Moldova2017/1	Moldova	2017	99,99%	100%
Arm/07/CBM/c2	Armenia	2007	99,99%	100%
Hanoi_2019	Vietnam	2019	99,99%	100%
Korea/pig/Yeoncheon1/2019	Korea	2019	99,98%	100%
CzechRepublic2017/1	Czech Republic	2017	99,98%	100%
Belgium2018/1	Belgium	2018	99,98%	100%
Pig/HLJ/2018	China (index case)	2018	99,98%	100%
HU_2018	China	2018	99,98%	100%
Timor-Leste/2019/1	Timor Leste	2019	99,98%	100%
Belgium/Etalle/wb/2018	Belgium	2018	99,98%	100%
Pol18_28298_O111	Poland	2018	99,98%	100%
ASFV/pig/China/CAS19-01/2019	China	2019	99,98%	100%
China/2018/AnhuiXCGQ	China	2018	99,98%	100%
ASFV-wbBS01	China	2018	99,98%	100%
ASFV/POL/2015/Podlaskie	Poland	2015	99,98%	100%
Pol17_31177_O81	Poland	2017	99,98%	100%
CN/2019/InnerMongolia-AES01	Mongolia	2019	99,98%	100%
ASFV/LT14/1490	Lithuania2	2014	99,98%	100%
GZ201801	China	2018	99,98%	100%
ASFV/Kabardino-Balkaria19/WB-964	Russia	2019	99,98%	100%
ASFV/Amur19/WB-6905	Russia	2019	99,98%	100%
NgheAn_2019	Vietnam	2019	99,98%	98%
VNUA--05L1/HaNam/VN/2020	Vietnam	2020	99,98%	98%
Estonia2014	Estonia	2014	99,98%	94%
Germany2020/1	Germany	2020	99,97%	100%
Wuhan2019-1	Wuhan	2019	99,97%	100%

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Pol19_53050_C1959/19	Poland	2019	99,97%	100%
Pol17_55892_C754	Poland	2017	99,97%	100%
ASFV/Primorsky19/WB-6723	Russia	2019	99,97%	100%
ASFV/Ulyanovsk19/WB-5699	Russia	2019	99,97%	100%
CADC_HN09	China	2019	99,96%	100%
VN/HY-1(2019)	Vietnam	2019	99,96%	99%
VN/QP-1(2019),completegenome	Vietnam	2019	99,96%	99%
Georgia2008/2	Georgia	2008	99,96%	99%
HuB20	China	2020	99,96%	99%
MAL/19/Karonga,completegenome	Tanzania	2019	99,95%	96%
Pig/Heilongjiang/HRB1/2020	China	2020	99,94%	100%
Tanzania/Rukwa/2017/1	Tanzania	2017	99,94%	96%
Georgia2008/1	Georgia	2018	99,92%	99%
ASFV/Kyiv/2016/131	Ukraine	2016	99,88%	100%

Analysis of different variable regions of the genome located in the central conserved region is used to differentiate between closely related isolates (Qu *et al.*, 2022). Among them, the most used are the tandem repeat sequences (TRS) located in the central variable region (CVR) of the *B602L* gene, and the intergenic region (IGR) between the I73R and I329L genes (Gallardo *et al.*, 2014). These regions are of particular interest due to the size difference that are directly visible by electrophoresis. By analyzing the IGR, two different variants were identified in Europe in 2014, making it possible to trace the origin of the EU outbreaks from Lithuania and Poland. These isolates were classified within the IGR2 variant, characterized by the insertion of an TRS, present in isolates from bordering countries such as Belarus, but different to the Georgia index case (Gallardo *et al.*, 2014). The molecular characterization carried out in the EU reference laboratory of ASF (EURL, Madrid, Spain) of more than 2,600 ASFVs from both wild boar and domestic pig obtained between 2014 and 2022 in the EU, has classified the EU genotype viruses in four IGR variants (IGR1 to IGR4), with the IGR2 variant being the predominant (C. Gallardo, personal communication June 2021; Mazur-Panasiuk *et al.*, 2020). The analysis of the TRS of the CVR has identified only two variants among the genotype II viruses of the EU, although the CVR variant 2 was temporarily restricted from 2015 and 2016 within the wild boar population in southern Estonia (Vilem et al., 2020). The CVR1 variant (Georgia07 type) is predominant. Analysis of other regions, such as the *O174L* gene, has also made it possible to identify two variants that circulate simultaneously in Poland, with variant 2 characterized by the additional insertion of 14 nt, which represents a tandem repeat (Mazur-Panasiuk et al,, 2020). It is important to note that 95% of reported cases in the EU since 2014 have occurred in wild boar, and the virus, depending on the ecological context, may persist in wild boar populations with or without reintroduction of infected domestic animals. Therefore, the variations in the TRS could be related to a spontaneous mutation caused by the maintenance of ASFV within the wild boar population in certain regions of the EU. IGR sequencing between the *9R* and *10R* genes of MGF505 supported this hypothesis with seven MGF variants identified from wild boar genotype II viruses circulating in Europe (C. Gallardo personal communication 2021).

The virus causing the outbreaks in China was initially classified as genotype II, CVR1, IGR-II predominant in Europe (Ge et al., 2018). Whole genome sequencing showed the highest sequence homology with a Polish isolate from 2015, but due to lack of information on circulating Russian ASFV isolates, the origin of the outbreak cannot be elucidated (Bao *et al.*, 2019). Since 2018, different genetic IGR variants (IGR 1 to IGR 4) have been detected circulating in both domestic pigs and wild boar in China, Korea and Vietnam. But without a doubt the most important fact has been the identification of genotype I of ASFV in China in July 2021, specifically in pig farms located in the provinces of Henan and Shandong (Sun *et al.*, 2021a). The phylogenetic analysis of the complete genome sequences showed a great similarity with the ASFV NH/P68 and OURT88/3, two genotype I attenuated ASFVs isolated in Portugal in the last century. It is important to note that animals infected with the Chinese genotype I virus developed a chronic disease that could go unnoticed in the field due to its reduced virulence (Sun *et al.*, 2021a). The source of these viruses and the nature of their introduction into China is unclear. Although they may represent a new introduction of the virus from an African source, the striking degree of genetic similarity to Portugal isolates from the 1960s suggests that they may have originated from other source, possibly imported legally or illegally for evaluation as candidate vaccine. The appearance of this genotype I presents more problems and challenges for the control and prevention of ASFV in Asia.

In summary, current genotype II ASFV strains affecting Europe and Asia are closely related and share more than 99% homology when whole genome sequences are compared. The implementation of a subtyping method in routine diagnosis is necessary to identify the origin of an outbreak and the dynamics of the disease in the affected countries. Due to the low mutation rate of being a DNA virus, the utility of a single method is limited and allows only moderate discrimination of closely related strains. The use of a standardized protocol using multiple genetic markers needs to be investigated and implemented internationally. Furthermore, since current genetic characterization approaches are not related to biological properties (Arias *et al.*, 2018), it is necessary to identify new genetic markers that can explain changes in virulence without the need for experimental infections. The genetic characterization of genes involved in

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virulence such as the MGF505-7R gene (Li *et al.*, 2021) and the EP402R (CD2v) could be a potentially interesting area of research.

Biological properties of genotype II ASFVs.

ASFV strains induce variable clinical forms, ranging from acute and peracute infections with a mortality of 90 to 100%, to subacute and chronic forms with much lower mortality (Salguero *et al.*, 2020). ASFV genotype II strains circulating in Europe and Asia are generally virulent causing acute disease with almost 100% lethality in both domestic pigs and wild boar. After infection with virulent strains, animals develop acute clinical signs between 3.5 and 14 dpi (mean) and 91 to 100% of animals die between 7 and 25 days (Pikalo *et* al., 2019). However, one of the greatest concerns in recent years in Europe and Asia is the continuing trend towards the appearance of clinically milder or completely unapparent forms in endemic areas of the disease. This attenuated phenotype was initially detected in Estonia in 2014 in the north of the country. Experimental *in vivo* studies showed that the ASFV isolated in 2014 in north-eastern Estonia (Ida-Viru region) was moderately virulent in domestic pigs, but remained highly virulent in adult wild boar. Sequencing detected a 14.5 kb pair deletion at the 5' end responsible for the attenuated phenotype (Nurmoja *et* al., 2017; Zani *et al.*, 2018). A year later, in 2015, moderately virulent strains were identified in southern Estonia (Gallardo *et al.*, 2018). Pigs experimentally infected with these strains presented variable symptoms that included acute, subacute and chronic forms of ASF. 33% of the animals survived the infection with mild and nonspecific symptoms from day 14 to day 30. After a period of apparent recovery, clinical signs reappeared two months later (50-60 days) and were similar to those described with other moderately virulent strains of the genotype I (Malta and DR78). Surviving pigs had intermittent viremia lasting up to 78 days, although infectious virus was only recovered from blood for approximately one month. These animals showed a positive antibody response from the second week, which was maintained until the animals were sacrificed (2-4 months). In Poland, the presence of less virulent isolates has also been described (Walczak *et al.*, 2020). Following intranasal infection of domestic pigs with ASFV Pol18_28298_O1, the animals developed various forms of the disease (acute, subacute and chronic) and mortality ranged from 80 to 100% depending on the dose. Two pigs survived the infection with nonspecific clinical signs, no fever and short viremia, but high antibody response.

In 2017, the first attenuated, non-haemoadsorbent (HAD) genotype II ASFV strain, Lv17/WB/Rie1, was isolated from a wild boar hunted in Latvia (Gallardo *et* al., 2019a). The HAD phenomenon consists of the adsorption of red blood cells around monocytes/macrophages that have been infected by ASFV. The image under the microscope is presented in the form of a morula or crown (rosette) of erythrocytes around the infected cells. The effect of HAD is related to the presence of two genes in the ASFV genome, the EP402R ORF that encodes a protein homologous to the molecular surface marker CD2, and the EP153R gene that encodes a protein homologous to the molecular surface marker CD44. The first is responsible for the HAD phenomenon because when the virus replicates in target cells, it expresses CD2 as a surface marker, producing the union between the CD58 present on the surface of the erythrocytes and the CD2 of the infected cells. The union, which gives rise to a morula or rosette-shaped structure, is stabilized by the action of the protein encoded by the EP153R gene (figure 2).
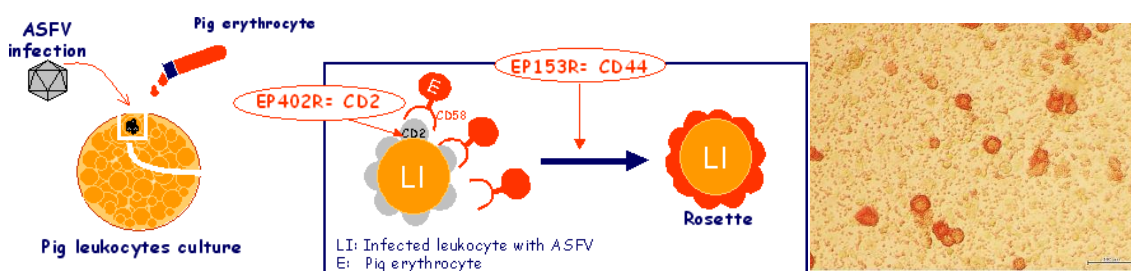
Figure 2: schematic representation of the HAD phenomenon after ASFV infection

In the ASFV Latvian strain, sequence analysis of the *EP402R* gene revealed a single mutation that generates a truncated protein that encodes a non-functional truncated protein and thus loses HAD capability. This feature is shared with other field-attenuated ASFV strains, such as NH/P68 and OURT88/3, or the recently discovered non-HAD genotype I Chinese ASFVs (Sun *et* al., 2021b). Pigs experimentally infected with the non-HAD ASFV Lv/17/WB/Rie1 developed non-specific clinical signs and, in some cases, remained asymptomatic, showing weak and intermittent viremia and a high antibody response. Of note, the virus genome was detected in a pig in 42% of tissues tested and African swine fever virus was isolated from retropharyngeal and submandibular lymph nodes, primary replication sites, at 101 days, i.e. that is, more than 3 months after the primary infection. Since the first description in 2017, eleven non-HAD genotype II ASFVs have been isolated from wild boar in the EU, in Latvia and in Poland (Gallardo. C, unpublished data 2021). Notably, non-HAD Polish viruses were obtained from wild boar hunted near the border with Kaliningrad (an enclave of Russia) or, in the case of Pol18/WB/Case1865, just 20 km from the border with Belarus. (Figure 3A). These data seem to suggest that these viruses are circulating in wild boar through natural

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corridors between countries and are not restricted to EU countries only. However, the lack of information on viruses circulating outside the EU does not allow us to support this hypothesis. Latvian viruses were isolated from nearby locations where the first non-HAD virus was detected in 2017 (Figure 3B).
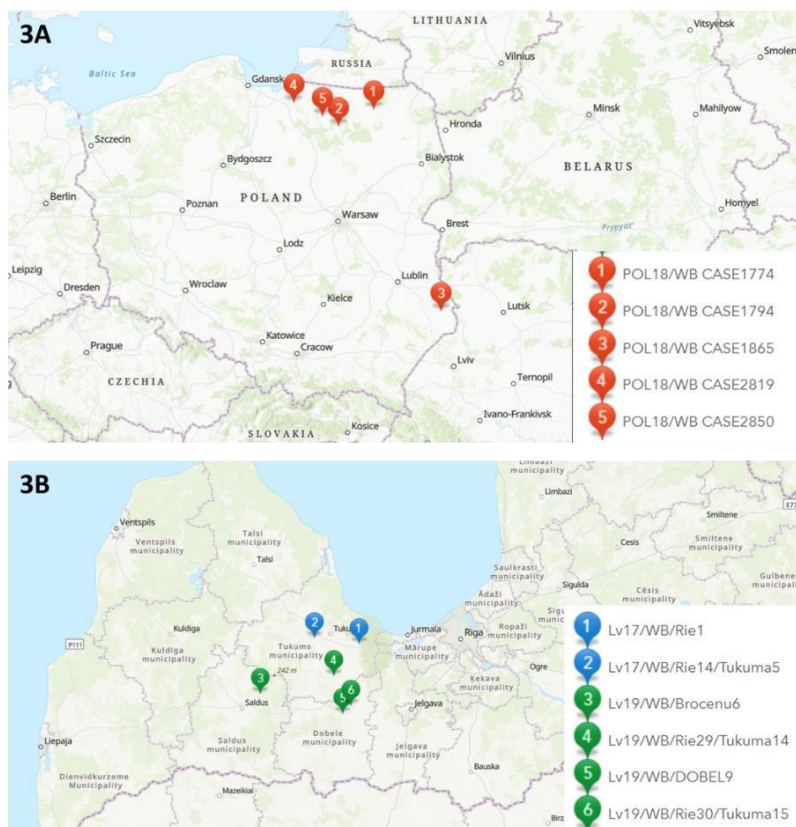

Figure 3: schematic representation of the HAD phenomenon after ASFV infection

Similarly to that described for the Lv17/WB/Rie1, the eleven non-HAD ASFV isolates had different types of mutations or deletions in the *EP402R* gene that prevent the viruses from translating intact CD2v protein and result in a non-HAD phenotype. When tested in domestic pigs, they induced subacute or chronic disease, or even some pigs remained asymptomatic (C. Gallardo unpublished data). As reported in the EU, 11 non-HAD viruses were isolated in China during a surveillance program conducted from June to December 2020 (Sun *et al.*, 2021b). Chinese non-HAD viruses had four different types of naturally occurring mutations or deletions in the *EP402R* gene and showed lower virulence in domestic pigs, but were highly transmissible similar to that seen with EU ASFVs.

In conclusion, numerous studies have shown the natural evolution in Europe and Asia of ASFV genotype II towards less virulent forms circulating together with virulent viruses. The clinical manifestations of the disease are more variable and more difficult to recognize in the field. The infection may persist for several months with no particular symptoms evident in infected animals, apart from stunted growth or emaciation, or may even mimic other diseases. Although a long-term carrier state has not yet been experimentally demonstrated, the question is whether these animals that survive the infection have the capacity to infect a naïve population and whether or not they play an important role in the epidemiology of the disease. That is, in persistence of the virus in endemic areas, the appearance of sporadic outbreaks and the introduction into new regions. It cannot be excluded that a very small number of animals can transmit ASFV even in the absence of virus presence in blood and thus maintain the virus in endemic areas.

Animals surviving acute and subacute infections shed virus in oral secretions for up to approximately 22-30 days and in blood for up to 44-60 days. Therefore, an infected animal would act as a carrier of the virus during this period, although it would remain asymptomatic (Blome *et al.*, 2020; Gallardo *et al.*, 2018, 2021; Sun *et al.*, 2021a, b; Walczak *e*t al., 2020; Zani *et al.*, 2018;). The risk of oral transmission in pigs infected with attenuated isolates is much lower than in the case of infections with strains of high or moderate virulence, although this circumstance can never be ruled out. It has been experimentally demonstrated that seropositive animals infected with attenuated strains can transmit the virus to a susceptible population, more than three months after infection, in the absence of viremia or clinical signs (Gallardo *et al.*, 2015). These results contradict the assertion by some investigators that the probability of a seropositive but virus-negative "survivor animal" shedding infectious virus and playing a role as a carrier is virtually zero (Schulz *et* al., 2021).

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An important factor to take into account is the type of samples that are analyzed in epidemiological surveillance programs. Both the matrix used and the quality of the samples can influence the probability of detecting low numbers of viral genomes or that the virus is confined to a specific organ that is not included in the programs. In this sense, the detection of various tissues could help the detection of possible virus carriers. In surviving animals and those that develop chronic or subclinical infections, the virus is cleared more or less rapidly from target organs such as bone marrow, spleen, or kidneys. However, it persists for more than two months, and has even been detected at 4 months, in primary replication sites, such as tonsils and lymph nodes, or in secondary replication sites, such as intra-articular tissues (Gallardo *et* al., 2015, 2019a; 2021; Walczak *et* al., 2020). Presence at primary replication sites, which occur to some extent in any of the survivor categories, could suggest the likelihood of persistent infection or pigs having multiple reinfections with the same strain, as the virus is usually present where replication occurs. Taken together, these data suggest that other tissues should also be considered as target samples in surveillance programs. Furthermore, the question of whether ASFV present in a tissue could be reactivated in seropositive animals under immunosuppression, stress or in case of death must be investigated. To find scientific evidence on these issues, long-term experimental studies will be unavoidable.

ASF diagnosis. state of the art, gaps and priorities

Since there is no vaccine available, the prevention, control and eradication of ASF depends on the implementation of adequate surveillance that detects outbreaks as early as possible, as well as the ability to respond quickly and efficiently to prevent its spread. A key element is early detection. This is important for any infectious disease, but even more so for ASFV, because the virus survives for long periods in the environment and in pork products, and because there are different co-circulating strains. Designing a sufficiently sensitive ASF surveillance system requires a sound understanding of the epidemiology, the virus and the disease, along with an adequate diagnostic laboratory. A wide spectrum of accurate diagnostic tests for ASF is available and most of them have been used successfully in surveillance, control and eradication programs (Gallardo et al., 2019b). However, as in any other disease, there is no single test that is 100% reliable (sensitive and specific). For this reason, the final diagnosis must be based on the interpretation of the results derived from the use of a series of validated tests on the appropriate samples, in combination with information from the epidemiology of the disease and clinical signs.

ASF diagnostic workflow.

In case of ASF suspicious, PCR is by far the method of choice. It is fast and can be used for individual and pooled samples, albeit with size limitations (Gallardo et al., 2019b). There is a wide range of validated PCR tests, both commercial and *"in house"* that can be used at the laboratories providing a confident diagnosis (Auer *et* al., 2022; Gallardo *et* al., 2019b; Pikalo *et* al., 2022). A recently described real-time duplex PCR for rapid detection and differentiation between ASFV genotypes I and II provides an additional powerful tool that may facilitate efficient control of ASFV in regions where both genotypes may be present (Li *et* al., 2022). However, it must be taken into account that, although sporadically, false positives can be obtained in PCR (for example, due to laboratory contamination or other factors). Therefore, in the case of a primary outbreak (or a wild boar case), whenever there is a positive result for ASF, this should be confirmed by an alternative virus or antibody detection technique. The ideal is to isolate the virus and perform the HAD technique to establish whether the virus is HAD or not, and to perform the genotyping to determine the origin of the outbreak. However, this may not always be possible due to technical limitations, lack of adequate facilities, or reduced sensitivity, particularly in samples obtained from hunted wild boar, or in weak positive PCR samples (Gallardo *et* al., 2019b).

Whenever ASFV is suspected to be circulating in a pig population, a negative PCR result cannot exclude the presence of ASF. Since animals generally develop antibodies within the second week after infection, they can test positive for both ASFV and antibodies simultaneously for at least two months. Samples from animals surviving this period are usually positive for ASFV-specific antibodies, but negative for ASFV and its genome. Therefore, if the PCR was negative but ASFV is suspected to be circulating, serological tests should also be used for diagnosis. It should be emphasized that serology tests were essential in the ASF eradication program in Spain and made it possible to detect "potential carriers" (Arias *et* al., 2018). Current recommendations for the detection of antibodies to ASFV involve the use of an ELISA, supported by immunoblot (IB), indirect immunofluorescence test (IFAT), or indirect immunoperoxidase tests (IPT) as confirmatory tests (OIE 2021). ELISA remains the most useful method for large-scale serological studies on serum samples: it is fast, easy to perform, and inexpensive. However, only serum can be tested, which restricts its range of application, especially in the case of passive surveillance of wild boar when the animals are often found dead. In addition, hemolyzed serum samples could give false positive or false negative results depending on the ELISA format used. Therefore, positive ELISA results should always be confirmed by additional methods such as IPT, IFAT or IB tests, as recommended by the OIE (OIE 2021). The IB is a rapid and sensitive assay, but similar to that described above, only serum samples can be tested. In contrast, IPT or IFAT can be easily used to analyze all types of porcine samples, including tissue exudates, whole blood, fluids, and even bone marrow. Detection

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of antibodies by IPT in tissue samples of exudates is a common successful method when testing wild boar. In the figure 4 is summarized the workflow for ASF detection in case of suspicious.
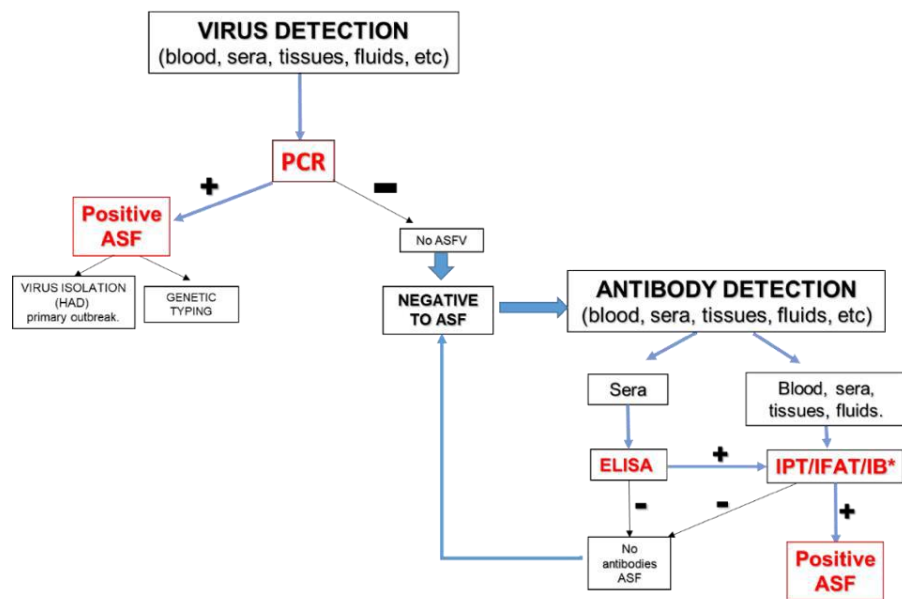

Figure 4: schematic representation of workflow of ASF diagnosis in case of suspicious. *Source: webpage of the ASF-EU reference laboratory (EURL);* https://asf-referencelab.info/asf/en/

The starting point for any laboratory investigation is the collection of samples. An important consideration is the purpose of the research, for example, disease diagnosis, disease surveillance, or health certification. The animals to be sampled will depend on the objective of the sampling. When investigating an outbreak (passive surveillance), sick and dead animals should be targeted, while in older animals should be sampled when checking to see if animals have been exposed to disease (active surveillance). To be effective, the appropriate samples combined with the selection of diagnostic methods are of fundamental importance to make a rapid and reliable diagnosis. Samples collected from live pigs should include anticoagulated whole blood for virus or viral nucleic acid detection and serum for antibody detection, while samples collected from dead pigs or wild boar should include tissues for virus and antibody detection (https://asf-referencelab.info/asf/images/GUIDELINES_DIAGNOSIS/Link_1_sampling_collection.pdf). However, these type of samples may not always be available, and alternative samples are required, especially for passive surveillance of wild boar and for active surveillance in large pig farms. Different types of alternative samples have been tested that could meet the objective of providing a reliable diagnosis. Published options include dried blood swabs, dried filter papers and FTA cards, fecal samples, oral, nasal, and rectal swabs, meat juice, various roped-based options, ear picks, or dry sponges (made by 3M) (Carlson *et* al., 2018; Flannery *et* al., 2020; Giménez-Lirola *et* al., 2016; Goonewardene *et* al., 2021; Kosowska *et* al., 2021; Mur *et* al., 2013; Onyilagha *et* al., 2021; Pikalo *et* al., 2021; Randriamparany et al., 2016). Comparative studies of such alternative samples *vs.* classical samples confirmed that blood continues to be the most suitable option for the detection of viruses and antibodies, together with sera, both in the early and late phases. Alternative samples, such as blood swabs or oropharyngeal swabs, have been shown to be the most promising alternative samples although susceptibility results depend on the virulence of the strain. When samples are collected within the first three weeks of infection (≈ 3-20 days), the highest proportion of PCR-positive samples is obtained from oropharyngeal swabs, regardless of strain. On the contrary, in the blood samples there are significant differences. While in acute and subacute infections the virus is detected in similar proportions in blood and in oropharyngeal swabs, in animals with chronic or subclinical disease, viremia peaks are intermittent, even at the beginning of the infection. As of day 20, the ASFV genome is only detected sporadically in these animals and in a clearly lower percentage than that detected in oropharyngeal samples (Gallardo *et* al., 2021). However, in animals that survive infection caused by moderately virulent isolates, the ASFV genome can be detected in blood for a period of about two months or even up to 100 days (Blome *et* al., 2020), while in oropharyngeal samples the detection range is usually lower at one month (Gallardo *et* al. 2021). Therefore, the oropharyngeal swabs should not be used as a substitute for blood in active surveillance, as it would decrease the detection of animals that survived to the primary infection with either virulence or moderate virulence ASFVs, as they would possibly not be showing any clinical signs. But together with blood, even with blood swabs, the oropharyngeal swabs samples could allow to detect ASFV infection for a longer period and could be a useful an alternative sample in the passive surveillance programs for the early detection prior to onset of obvious clinical signs, mainly in large pig farms (Gallardo *et* al., 2021; Pikalo *et* al., 2021). In conclusion, alternative approaches are feasible, but should be integrated into control strategies by selecting test methods and sample materials following a "fit for purpose" approach.

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ASF diagnosis future trends.

A large number of validated ASF diagnostic techniques are currently available to provide a reliable diagnosis of ASF in affected countries (Gallardo *et al.*, 2019b). However, there is a strong demand for accurate, fast and simple detection methods, especially for on-site applications. Several approaches for rapid nucleic acid detection, including molecular platforms are now available that could allow sensitive detection of ASFV DNA, mainly in the early stages of the disease. These tests can also be used to detect contaminated carcasses, pork, and environmental samples at the point of need (e.g., slaughterhouse, airport, or boar/wild boar habitats). However, these platforms are technically more complex than rapid antibody or antigen tests and require more field validation studies and a much higher level of training and competence to perform accurate tests. Molecular field tests also require expensive equipment for amplification and, in many cases, extraction of viral DNA. Regarding the antigen detection test, despite not being included in the Registry of Diagnostic Kits certified by the OIE as validated as suitable for use, there are several Point of care (PoC) tests, which are commercially available for testing including basic rapid tests using lateral flow devices (LFDs). These tests are easy to use, require minimal training, and can provide a result in about 20 minutes. Rapid antigen tests are generally less sensitive than molecular techniques for virus detection, but some may have comparable levels of specificity. The use of antigen tests in symptomatic and terminally ill pigs that have high levels of viremia is recommended, rather than pigs in the early stages of clinical infection that may not have a high enough viremia to allow detection. It is recommended that samples from more than one sick pig be tested to increase the chances of detecting infection. LFDs for antibody detection generally have comparable levels of sensitivity and specificity to laboratory ELISAs, although they show lower sensitivity compared to reference tests such as IPT. LFDs can be used to detect antibodies in pigs that have survived infection although, as with LFD antigen, both types of tests should be used in parallel to avoid losing infected animals. The choice of which PoC method to use can be influenced by many factors including costs, ease of use and training requirements. Simple rapid tests may be appropriate for certain situations, such as resource poor settings, while more advanced molecular platforms may be selected in settings where costs are not a major factor and operators can be confidently trained to a high level of competency. For some countries, a combination of tests may be employed depending on application and available resources. The OIE ASF Reference Laboratory Network's has created an overview of African swine fever tests for field application, a document that summarise current knowledge on commercially available field tests, including a range of technical details, cost, as well as advantages and disadvantages of each. It is available at https://www.oie.int/en/document/the-oie-asf-reference-laboratory-networks-overview-of-african-swine-fever-diagnostic-tests-for-field-application/

A major drawback in the diagnosis of ASFV is the isolation of the virus due to the lack of appropriate established cell lines. Virus isolation from field samples relies on primary cell cultures, a costly procedure and requires specialized facilities, training, and time. In 2020, the cell line MA-104R (a commercially available African green monkey kidney epithelial cell line) was identified for the isolation of several ASFV strains (Rai *et al.* 2020, 2021). It allows to identify if an isolate is HAD or not and to isolate the virus directly from clinical field samples. However, additional studies are needed to determine if the viruses isolated in this cell type undergo genetic modifications when carrying out consecutive passages. There are other cell types derived from porcine macrophages such as IPKM (Masujin *et al*, 2021) or ZMAC-4 (Portugal *et al*, 2020) that are also capable of efficiently replicating different ASFV isolates. However, these cells require further investigation to verify whether the virus is isolated directly from clinical samples, without adaptation process, and maintains productive viral replication. These studies should be aimed at validating the recently described cell lines with clinical samples and their suitability for isolating ASFV without inducing genetic and/or phenotypic changes.

Other important gap is that available ELISAs against anti-ASFV antibodies are only suitable for serum samples, which limits their applicability, especially in endemic areas that lack standardized wild boar sample collection programs (Gallardo, *et al.*, 2019b). This problem is overcome today with the use of IPT, which can easily analyze all types of samples, such as blood and exudates from tissue samples, including bone marrow. However, this technique is not commercially produced by companies, which restricts its use in laboratories, especially those with limited resources. In this context, standardized ELISAs for the detection of specific antibodies in tissue extracts or blood are needed for an easy and more reliable assessment of the epidemiological situation in the affected areas. The INgezim® ASFV-R ELISA technique has been validated for the detection of specific antibodies in serum, blood (fresh or on paper) and spleen exudate samples from pigs and wild boar, with blood being the best target sample. This is especially interesting in endemic areas where strains of low virulence circulate and viruses in organs are not easily detected. These animals have a high titer of antibodies in their tissues (Gallardo *et al.*, 2018, 2019a, 2021) and therefore could be easily detected by the ELISA test, providing a more detailed picture of the epidemiological situation in endemic areas.

Concluding remarks

ASFV is a complex DNA virus that has a significant impact on the global swine industry. The lack of a safe and effective vaccine and the reliance on herd culling to prevent the spread of the disease has resulted in significant

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economic losses. Therefore, improved early detection remains a significant priority. Taken together, sensitive, specific and robust laboratory diagnostic assays are available but, as for any other disease, there is not a single test being 100% reliable (sensitive and specific). For this reason, final diagnosis should be based on the interpretation of the results derived from the use of appropriate samples and validated tests in combination with the information coming from disease epidemiology, the presence of clinical signs and the scenario. A thorough understanding of the viremia and antibody seroconversion timing during ASFV infection is a prerequisite to conclude the dynamic of the infection in the investigated areas, and to support control and eradication programs. Positive results for both virus and antibodies indicate that the tested animal was infected at the time of sampling, whereas a positive ASFV antibody test in absence of virus indicates an ongoing or past infection, where the animal has recovered or could be chronically or sub-clinically infected with attenuated strain. These animals should be detected since they can act as carrier of the virus and, in certain conditions to infect a naive population. On the international level, laboratory methods as well as sampling and shipping guidelines can be found in the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 3.9.1, version adopted in May 2021). The selection of which test to use depends on available matrices, the purpose of the testing (surveillance, eradication, diagnosis, confirmation), as well as the ASF epidemiological status of the country (region) or stage of the epidemic in the region.

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Vaccines and vaccination in PRRS

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Introduction

Since its emergence in 1980s, porcine reproductive and respiratory syndrome (PRRS) has become one of the costliest diseases for the swine industry. Control of infection is based on four pillars: biosecurity, management of the herd, diagnosis and monitoring, and immunization.

The first vaccines against PRRS virus (PRRSV) were developed and launched on the market in the 1990s. Soon it became evident that vaccines were a valuable and necessary tool for the control of the infection, but that was limited. Although vaccines reduce the clinical and economic impact of the disease, vaccinated animals could still be infected. The genetic and antigenic diversity of the virus, the limited knowledge on the correlates of protection, the unclear nature of heterologous protection, and the wide diffusion of the virus in high pig-density areas are obstacles for achieving greater efficacy. Moreover, the development of novel vaccines using more advanced technologies is limited by the acceptable cost of vaccines for swine production. In the present lecture, we will review the main elements that allow the understanding of what can be expected from current vaccines and what can be expected in the future.

Characteristics of currently available vaccines

At present, most of the commercially available vaccines against PRRSV are made of modified-live (ML) PRRSV with or without adjuvants. Less commonly, inactivated vaccines (IV) are also used. The published evidence indicates that live vaccines are required for an effective primo-immunization (1,2), while inactivated vaccines are mostly used for recall purposes (3). However, the inactivation procedure and the adjuvant addition may result in different efficacies of the inactivated vaccines. For example, Vanhee et al. (2009) showed that a BEI-inactivated vaccine was able to induce neutralizing antibodies. Geldhof et al. (2012) indicated that autogenous BEI-inactivated vaccines could be useful to induce a more or less predictable immunity against a given variant. Other authors (6), suggested an important role of adjuvants for the development of immunity when inactivated vaccines are used.

Attenuation of PRRSV used in vaccine products is achieved by serial passage in non-host cell lines, resulting in variants that have a lower replicative capacity _in vivo_. The specific mechanisms leading to attenuation are not well known and this is a problem for the rationale development of fully safe attenuated vaccines. Several strategies have been used to develop attenuated PRRS viruses that could be potentially used in new vaccines (reviewed by Wang et al., 2020). These strategies included modification of nsp1β, nsp4, nsp2TF and nsp2N, production of chimeric viruses, codon de-optimization and others. However, a review of the literature shows that virulence of a given PRRSV strain has been associated at least with the mentioned proteins plus nsp2, nsp9, nsp10, GP2, GP3, GP5 and M. In summary, virulence is most probably multigenic and involves mechanisms related to replication, to the induction or inhibition of inflammatory responses and the development of specific immunity and apoptosis. At present it is difficult to foresee a single strategy leading to a universal mechanism of attenuation for all PRRSV strains.

Another consideration with PRRS MLV is the production in cell cultures that express alternate receptors for the virus. On one hand, the adaptation of the virus to use an alternate receptor may contribute to attenuation but, on the hand, it might also imply a lower replication in the host or the induction of immune responses different (in either intensity or quality) to those induced by the natural infection. With the aim to overcome this, a recently marketed vaccine is produced in a CD163-transfected cell line (8). Notwithstanding, in general, the on-field efficacy of all MLV seem to be very similar.

Considering modified live vaccines (MLV) as the current standard for PRRS vaccination, it is possible to draw a pattern of immune response that can represent most situations. Naïve pigs vaccinated with an MLV quickly (7-21 days) develop antibodies but those early antibodies are devoid of neutralizing capability. Neutralizing antibodies are developed after 4-5 weeks, and, in general, with a single administration the neutralization titers are low (1,9). An important point here is whether these neutralizing antibodies induced by the vaccine result in real protection in on-field situations. Several studies have shown that the ability of the antibodies induced by one PRRSV strain for neutralizing other strains is the result of at least four factors, the immunizing strain, the susceptibility to neutralization of the infecting strain, the mutations that could potentially arise in the course of the infection and the idiosyncrasy of

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each pig (10-13). Two experimental studies showed that passive transfer of homologous neutralizing antibodies (titers 1:8-1:16) induce protection against the homologous infection in both the sow and the piglet models (14,15). However, forecasting the extent at what the neutralizing antibodies induced by one vaccine may actually protect against a given field strain is not possible. We have been examining sera of sows vaccinated with commercial PRRSV1 MLVs for the capability of neutralizing several PRRSV1strains circulating in Spain. Sera of gilts that never had any contact with PRRSV other than the MLV were unable to neutralize contemporary field strains at titers higher than 1:4. Although obtained from a limited sample, these results suggest that pre-formed neutralizing antibodies induced by vaccination could have a secondary role in the initial protection against infection. However, the priming induced by vaccination could be useful to produce a secondary humoral response that can contribute to a faster clearing of the infection. See Rahe & Murtaugh (2017) for a review on the effector mechanisms of the humoral response to PRRSV.

Cell-mediated immunity is usually considered essential for the clearing of viral infections. Most often, the frequencies of virus-specific interferon-gamma (IFN-γ) secreting cells (IFN-γ-SC) are measured using the ELISPOT technique (1,9). When these are examined after vaccination, significant responses are usually detected 3-4 weeks post-vaccination. However, the frequencies of these cells oscillate with highs and lows for several weeks until they reach stability (1,9). Cell-mediated responses may be responsible for part of the heterologous protection (1,10). Correas et al. (2017) tested the cross reactivity of IFN-γ SC among PRRSV2 isolates and found a broad crops reactivity regardless the genetic distance between them. The cell-mediated mechanisms correlating with protection and the T-cell subsets involved in them are not fully clear. Chung et al. (2018) showed that PRRSV-recovered animals develop cytotoxic T-cells that, surprisingly were found with the CD4+ subsets. In another recent study (19) it was suggested that γδ T-cells may play an essential role cells in the anti-PRRSV response in the lymphatic system. We have recently followed a cohort of vaccinated gilts (2 x MLV before first mating) that were entered into a farm that was infected by PRRSV. The gilts that suffered reproductive problems leading to transplacental infection had a lower frequency of IFN-γ-SC cells among CD4+ cells. Taken together, all these results point towards CD4+ T-cells and γδ T-cells as the main T-cell subsets involved in protection and suggest that individual variability in the response to vaccination can also be important.

Vaccination of gilts and sows has been shown efficient in limiting clinical signs (reduce the probability of abortion, stillbirths, etc.), although it may not fully prevent vertical transmission (20, 21). The main objectives of vaccination programs for sows are to reduce the clinical and economic impact as well as to reduce the frequency of vertical transmission events. The starting point of vaccination programs for breeders is immunization of gilts. Usually, gilts are vaccinated twice with an MLV before the first mating to ensure immunity before entering in the sow herd. Once in the herd, recall vaccinations are needed in order to maintain immunity. In general, recall doses are administered every third or fourth month (blanket vaccinations 3-4 times/year) or are administered in a classical 6-60 protocol (that is two doses, the last at 60 days of gestation). While the first strategy is sought to produce a homogeneous immunity in the herd (all sows are vaccinated the same day), the second is intended to reinforce immunity before entering in the critical period for the transplacental infection (the last 30 days of gestation). However, in some sows, repeated administration of vaccines may not result in significant increases of the antibody titers (22). In another study, multiply vaccinated sows were examined for their serological status after and before vaccination (23). Between 3% and 19% of the multiply vaccinated sows that received an MLV recall dose remained or became seronegative (ELISA) after vaccination. In 5/171 examined animals (2.9%) a PRRSV-specific immune response could not be detected neither by ELISA, nor immunofluorescence, viral neutralization test nor ELISPOT. The authors suggested that some animals may become unresponsive or anergic after repeated vaccination with an MLV.

It is important to emphasize here that vaccination is a tool that must be accompanied by other interventions. Vaccination alone will seldom result in stabilization of the farm if there is a constant introduction of new PRRSV strain from other sources or if the farm is managed in a continuous flow system. Therefore, to be successful, vaccination programs for breeders must be combined with adequate biosecurity strategies and a correct management of the pig flow. Quarantines and PRRSV-free semen are essential elements of such a plan.

In piglets, vaccination with PRRSV1 MLV has been proven to have the potential for reducing transmission (as shown by a reduced R value in the vaccinated pigs) (24, 25). However, several considerations must be done. Firstly, those results were obtained under in experimental conditions in which the vaccinated animals were left to develop immunity after vaccination for several weeks. Secondly, only one challenge strain per experiment was tested and, thirdly, animals were devoid of maternally-derived antibodies (MDA) at the moment of vaccination. Considering that in real conditions the window for piglet vaccination is very narrow (in any case before or at weaning) and that the time between vaccination and exposure to the virus would be shorter than in the abovementioned experiments, efficacy for stopping transmission in the field may be lower. Actually, Chase-Topping et al. (2020) showed that under different circumstances, vaccination reduced R0 in comparison with the value in unvaccinated animals although this reduction did not lead to stopping the transmission among vaccinated animals. For PRRSV2, evidence on the reduction of the transmission has not been obtained.

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Interference with MDA may be an issue when vaccinating piglets. Several studies (27, 28) indicated that vaccination of piglets in the presence of maternally-derived neutralizing antibodies may result in partial blocking of the development of the immune response (both humoral and cellular) and decreased protection against challenge. Balasch et al. (2018) performed an experiment in which 1-day-old piglets with MDA were intramuscularly vaccinated with an MLV. Only 2/16 vaccinated pigs developed neutralizing antibodies, but they were protected from challenge with a heterologous strain. The authors concluded that protection was mainly produced by the cell-mediated immunity resulting from the vaccination.

The nature of such blocking is not fully clear. While neutralization of the virus by homologous or cross-reactive antibodies may effectively decrease the replication of the vaccine virus, and thus produce less antigenic stimulation, this is not the most common mechanism of blocking of MLV by MDA. In other models, blocking by MDA can be produced also by non-neutralizing antibodies. This is because the main mechanism involved in that blocking is the crosslinking resulting of the interaction of the complex antigen-antibody at the same time with the B-cell receptor and the FCγRIIB (reviewed by Nieswiek, 2014). As a result, cell-mediated immunity is usually little affected by MDA (30).

From early experiments, it was evident that PRRSV MLVs could be shed (32). Actually, the studies performed with PRRSV1 and PRRSV2 MLV indicate that detection of the vaccine virus in blood may continue for 3-4 weeks after vaccination with the consequent risk of shedding (33, 34). In sows, administration of an MLV in late gestation may result in vertical transmission and the birth of viremic piglets (although such transmission does not produce a significant impact on the production).

The wide use of PRRS MLV indicates their safety and that their efficacy outbalances the potential side effects. However, shedding the vaccine virus increases risks for virus recombination (35, 36) or reversion to virulence. Thus, rationale guidelines for using MLV must be applied when implementing a vaccination program. Some of the key elements of those guidelines are: a) Vaccination should be implemented only in infected herds; b) Vaccination should be applied only to pigs before the exposure avoiding the vaccination of already infected animals. An exception to this can be a blanket emergency vaccination to stop a reproductive outbreak; c) If an MLV is to be replaced by another a washout period should be observed to avoid the circulation of both vaccine strains in the farm, d) periodic monitoring of the herd by RT-PCR and sequencing will help to detect circulation of the vaccine or vaccine-derived viral clades and, e) a sudden increase in the incidence or apparent virulence of PRRS in vaccinated farms must be investigated. Such investigation must include sequencing of the causing strain and assessment of the potential participation of vaccine or vaccine-derived strains. The application of a rationale program of vaccination and a well-designed monitoring program will help to minimize the chances for recombination of vaccine viruses.

Future developments

In the last years, numerous papers described some additional PRRSV vaccine candidates, including subunit vaccines, viral-like particles, chimeric constructs, nsp2-deleted strains, etc. Unfortunately, up to date, none of them transformed into a commercial product. The main constraints for the development of new vaccines with universal coverage, or at least, capable of producing a strong immunity for most PRRSV1 or PRRSV2 strains are our incomplete understanding of the correlates of protection, the lack of a clear catalogue of relevant T and B-cell epitopes and the need for identifying adequate adjuvants or vehicles to potentiate the immune response.

New technologies and approaches may help to produce novel and more effective vaccines. Research in this area is intense. Among the newer vaccine technologies, nucleic acid vaccines, are undoubtedly rising stars. The success of RNA vaccines for SARS-CoV-2 transformed this technology from a subject of research papers into an everyday topic. Application of RNA vaccine technologies may help to develop tailored vaccines for some pathogens. In the case of PRRSV, the main problem again would be the selection of the protein or proteins to be included in such vaccines because of the lack of a clear knowledge of the critical epitopes and the multigenic nature of virulence. Other strategies may involve the use of replicative or non-replicative vectors. These strategies will face a similar problem. A different approach can be the use of PRRSV strains inducing broadly reactive antibodies: Again, the main obstacle is our incomplete understanding of how these broadly neutralizing antibodies develop and the importance of the individual response and the selected strain.

Moreover, another important issue is the time needed for the onset of the effective immunity and the duration of such immunity. In heavily endemic areas, exposure to the virus is almost granted as soon as the gilt exist the quarantine or the weaner enters the nursery. That means that inducing rapidly an effective immunity is very important. The number of doses required to achieve effective immunity is also important because this adds time and cost to the vaccination program. Finally, and no less important, the newer vaccines should represent areal breakthrough either because they produce universal protection (even in non-sterilizing) or because they produce enhanced protection (for example no significant transmission to or from vaccinated animals).

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At present, those objectives still seem to be very far. There is a need for increasing investment for understanding and identifying precisely the correlates of protection and the critical epitopes. The mechanisms leading to the development of broadly neutralizing antibodies and the bases of cross-protective cell-mediated immunity need to be further explored. Also, there is a lot to be done in the research on the role of individual genetic background and the development of the immune response.

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Classical swine fever vaccination of sows:
a means to prevent persistently infected offspring

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Classical swine fever (CSF) is one of the most important viral diseases in swine, having a large impact on the pig industries worldwide. While several countries with significant pig production are currently free from CSF, endemically infected areas remain, especially in Asia and parts of the Americas. In these countries, vaccination is usually applied to reduce the disease burden, but managerial problems and persistent infections can hamper control.

With regard to persistent infections, solid vaccination of sows before or during pregnancy is of great importance. Studies have shown that modern live marker vaccines are also able to protect against vertical transmission of the virus, especially if moderately virulent CSF virus (CSFV) strains are involved.

Apart from the long-known persistence that arises from intra-uterine infection, the phenomenon of postnatal persistence has been described to impact on vaccination efficiency. Postnatal persistence refers to a disease course that can be induced in piglets in the early hours and days of their lives. As persistence acquired during gestation, this course leads to constant shedding of CSFV, unspecific, mainly enteric clinical signs, and lacking antibody production. We could recently show that solid vaccination of the breeding sows, and thus transfer of maternal antibodies (MDA), can protect against induction of postnatal persistence. While naïve piglets developed postnatal persistence and showed tremendous viral genome loads and no detectable antibodies, MDA positive piglets were completely protected against clinical infection. No clinical signs were observed upon challenge. It was thus confirmed that solid vaccination of breeding sows can prevent the establishment of postnatal persistence in the offspring.

What more can genomic characterization tell us about pathogenic *Escherichia coli* in pigs?

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Introduction

All over the world *E. coli* is an important cause of a wide range of diseases in pigs, including postweaning diarrhea (PWD), edema disease, and septicemia (Fairbrother & Nadeau, 2019). Diarrhea due to *E. coli* may result in significant economic losses due to morbidity, mortality, decreased weight gain, and cost of treatment, vaccinations and feed supplements. Clinical signs fluctuate with time and regions and may range from mild diarrhea with 1.5 to 2% mortality and lower weight gain to severe diarrhea or sudden death with up to 25% mortality. PWD may be endemic or occur as outbreaks, and most commonly occurs in the first days through to several weeks after weaning and even after introduction into fattening herds. *E. coli* is the most common cause of PWD, although *Salmonella* and rotavirus may be associated with this disease and mixed infections may occur.

E. coli is a gram-negative bacterial rod that inhabits the intestinal microflora or ecosystem of most mammalian and bird species, including the pig. Most *E. coli* are commensals, that is, they reside in the intestine but are not harmful for the host animal. Only a small proportion of isolates are pathogenic, being classified into categories or pathotypes based on the production of broad classes of virulence factors and on the mechanisms by which they cause disease.
Strains of the most important pathotype in pigs, the enterotoxigenic *E. coli* (ETEC), produce one or several enterotoxins, which act on the intestinal epithelial cells to induce the secretion of water and electrolytes into the intestinal lumen, causing the clinical signs of diarrhea. The most important enterotoxins, which define ETEC, are the heat labile toxin LT and the heat stable toxins STa and STb. ETEC adhere to and colonise the intestinal mucosa via hair-like structures on the bacterial surface, called fimbriae. ETEC associated with postweaning diarrhea most commonly produce F4 (K88) or F18 fimbriae.

A second pathotype found in pigs with diarrhea is known as enteropathogenic *E. coli* (EPEC). EPEC were initially associated with diarrhea in children, especially in developing countries. These bacteria cause typical attaching and effacing lesions, and possess a variant of the EPEC attaching effacing factor Eae or Intimin.

Shiga toxin producing *E. coli* (STEC) produce one or more of a family of cytotoxins which are known collectively as Shiga toxins (Stx) or verotoxins (VT). In pigs, the most important STEC are those that cause edema disease. These strains produce the toxin variant Stx2e (VT2e) and the fimbriae F18. Certain isolates produce both Stx2e and enterotoxins, as well as the fimbriae F18. These isolates have been associated more with PWD than edema disease. Such isolates are designated as ETEC/STEC hybrids.

Extraintestinal pathogenic *E. coli* (ExPEC) are being increasingly associated with extraintestinal infections in swine, including septicaemia and respiratory diseases. These strains are not identified by the presence of a particular virulence factor but usually carry a large number of virulence genes that vary greatly between strains, and include at least one gene cluster for adherence, iron-binding and serum resistance.

Detection of pathogenic *E. coli* in pigs

Rapid detection and thorough identification of pathogenic *E. coli* permit an early, accurate diagnosis of disease outbreaks caused by these bacteria. This allows a timely and judicious choice of antimicrobial agent for effective treatment of affected animals and control of an outbreak. Accurate diagnosis also permits an informed decision for putting into place the most appropriate and efficacious preventive and control strategies, such as vaccination and management changes.

Currently, genotypic analysis such as polymerase chain reaction (PCR) is most commonly used to define the virotypes involved in an infection (Luppi et al., 2016). This test permits the detection of genes encoding for virulence factors such as toxins and adhesins. Primers recognising different genes related to toxins (STa, STb, and LT) and adhesins (F4, F5, F6, F18, F41, AIDA) for ETEC strains; attachment and effacement, such as eae for EPEC; Stx for STEC strains, and adhesins (P fimbriae), toxins (cytotoxic necrotizing factor (CNF)), and the aerobactin iron capturing system for ExPEC, are readily available and can be used to perform PCR.

Approach for complete identification of pathogenic *E. coli* in pigs

More complete characterization of isolates is performed by serotyping, and phylogenetic analysis using such techniques as pulse-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, amplified fragment length polymorphism (AFLP), ribotyping, 16S rDNA sequencing, traditional multi-locus sequence typing (MLST) based on seven house-keeping genes, multiple-locus variable-number tandem-repeat analysis (MLVA) and virulence factor analysis (Fratamico et al., 2016). This type of characterisation allows the monitoring of changing trends and the identification of new, emerging *E. coli* and virulence determinants that could gain importance due to the pressure of antimicrobial therapy. However, collectively, these tests are time-consuming and expensive and are more often carried out in surveillance programs and public health and research laboratories. Also, as these methods target fragments of the bacterial genome, they often fail to discriminate between closely related outbreak strains (Oniciuc et al., 2018).

Over recent years, whole genome sequencing (WGS) has emerged as an approach permitting a highly discriminatory genomic characterization of bacterial isolates. WGS is now widely used in research laboratories for the study of pathogenic *E. coli* and associated infections and is considered as one of the most promising techniques in clinical microbiology (Tagini & Greub, 2017). However, the cost of carrying out this test in the diagnostic laboratory is still relatively high, compared to the PCR tests which are routinely done for detection of pathogenic *E. coli*.

What does whole genome sequencing involve

Sequencing technologies have rapidly improved since a complete bacterial genome sequence was first reported in 1995 and several sequencing platforms are now available, allowing the sequencing of complete bacterial genomes within hours (Quainoo et al., 2017). Resulting fragmented DNA sequence reads are assembled into near complete genomes, which can be characterized using freely available web-based and command line software tools (Table 1). Several online platforms are available for analysis and visualization of sequencing data, which may be web-based or run locally (Uelze et al., 2020). An advantage of using such platforms is that little or no bioinformatics knowledge is required. The availability of more complete virulence gene databases is now allowing a more comprehensive characterization of *E. coli* isolates based on WGS data. WGS allows the detection of known virulence genes, but also permits the identification of new genes or their variants involved in bacterial virulence (Uelze et al., 2020). WGS typing methods, including core genome cgMLST and single nucleotide polymorphism (SNP) analysis, are being increasingly used and have a very high discriminatory power. Access to large databases to allow analysis of relationship of local isolates with others worldwide permits a more global management of outbreaks.

It is now within the capability of a veterinary diagnostic laboratory to carry out these analyses and provide a report of the results, either for the identification of individual isolates or of several isolates as part of an epidemiological study on a particular farm or group of farms. Such studies demonstrate the relatedness of isolates and the possible presence of clones and show transmission routes of clones, AMR resistance genes, or mobile genetic elements carrying AMR resistance and virulence genes. At EcL, we have been routinely offering this service for 3 years and it is a work constantly in progress as we gain experience and more user-friendly software tools and know-how become available. The present paper aims to address what is the added value of the use of WGS for epidemiological surveillance, outbreak detection and control of pathogenic *E. coli* infections in pigs, especially in a diagnostic context, for the veterinary practitioner.

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Table 1. Tools for different steps in the analysis of Whole Genome Sequencing data of athogenic *Escherichia coli* in pigs

Analysis step	Purpose of analysis	Analysis tools[*]
Genome characterization	Detect the presence of genes	Identification of bacterial species Identification of serotype Virulence genes AMR genes AMR mutations Biocide resistance genes Metal tolerance genes Identification markers for mobile genetic elements, e.g. plasmids
Comparative genomics	Detect relatedness between strains -are strains clonal? -what is outbreaksource?	SNP analysis Phylogroup Traditional MLST cgMLST wgMLST Kmers
Phylogeny	Determine routes of transmission of outbreak strains	Phylogenetic trees

[*]Quainoo et al. (2017)

What is the added value of Whole Genome Sequencing of pathogenic *Escherichia coli* in pigs and usefulness for the veterinarian

Table 2 summarizes the overall added value of carrying out WGS as opposed to the methods currently being used, and highlights the advantages for the veterinarian, either directly when it is carried out as a diagnostic test or indirectly when carried out in a surveillance or research context.

Table 2. Added value of Whole Genome Sequencing of pathogenic *Escherichia coli* in pigs and usefulness for the veterinarian

Added value	Advantages	Usefulness for veterinarian
Greater resolution in discrimination of related strains in epidemiological surveillance	Monitor AMR and mobile genetic elements Monitor emergence and transmission of clones Better understand the variation among isolates associated with disease	Indirect
Improved outbreak detection	Detection of emerging hybrid pathotypes Confirm ID of new pathogens Screen for particular traits Predict AMR	Direct
Improved infection control	Aid in preparation of autogenous vaccines	Direct

WGS permits better resolution in discrimination of related strains in epidemiological surveillance

Surveillance of AMR is currently based on phenotypic characterization of clinical, environmental, and food isolates, an approach which provides little information on mechanisms driving AMR or on the transmission of AMR genes and mutations throughout the food chain (Oniciuc et al., 2018). Use of WGS helps in the tracking of AMR genes and mutations and mobile genetic elements which carry these genes, identifying how they move through different production systems and ecosystems and their impact on the transmission of multiple AMR genes in bacteria infecting

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humans and pigs and other animal species (Wyrsch et al., 2016) (Table 2). Such an approach also allows the monitoring of the effect of withdrawal of antimicrobial usage and other strategies to reduce AMR such as administration of probiotics and bacteriophages, and the identification of routes of transmission of AMR on-farm (Duggett et al., 2020, De Lucia et al., 2021).

With respect to the monitoring of the emergence and transmission of clones, we demonstrated that a group of enrofloxacin-nonsusceptible ETEC:F4 isolates sharing at least 55% similarity based on their pulsed-field gel electrophoresis profile appeared in diseased pigs in Quebec in 2013 (de Lagarde et al., 2021). We further characterized these isolates using WGS to assess the possible presence of a clone. Prior to the mid-1990s, ETEC:F4 isolates from pigs in Quebec were mostly ST90 and belonged to several serotypes. Subsequently, isolates were mostly ST100, belonging to a single serotype, O149:H10. Clonal lineage A (ST100/O149:H10) was the most widespread and was composed of several clones. In particular, the presence of a high-risk ETEC:F4 clone not susceptible to enrofloxacin circulating in the swine population was demonstrated. Isolates belonging to this clone have a wide distribution, have been transmitted through the swine industry in North America, are multidrug resistant, possess specific virulence genes and have been able to persist for several months on the farm.

In another study on the possible emergence of new clones in a different geographical region, examination of isolates from postweaning diarrhea in Danish herds using WGS demonstrated the presence of a dominant clonal group for which the genome appears to have remained relatively stable since the 1990s, and is similar to isolates from China, the United States and Spain (Garcia, Gambino et al. 2020). Altogether, these results will help to guide in the choice of strategies to reduce the impact of postweaning diarrhea both at a local and global level.

WGS leads to improved outbreak detection

WGS is able to accurately predict pathotypes and to overcome the limitations of this classification as occurs with the emergence of strains with new pathogenic properties, such as hybrid pathotypes (Tagini & Greub, 2017) (Table 2). Thus, using WGS, it was recently shown that the ETEC and STEC virulence genes of a hybrid ETEC/STEC:F18 strain were carried on a single plasmid, suggesting a key role for plasmids in the emergence of hybrid pathotypes (Brilhante et al., 2019). Multiple resistance genes were carried on two conjugative plasmids on this same strain. Early identification of such emerging pathotypes permits rapid decisions in the choice of targeted treatment and control strategies to reduce the spreading of such plasmids.

More recently, a new more pathogenic edema disease-causing ETEC/STEC:F18 hybrid that appears to have originated from wildlife and not from domestic pigs was identified in wild boars using WGS (Perrat et al., 2022). This finding underlines the importance of monitoring of this hybrid to flag its possible transmission into domestic pigs.

Antibiotic susceptibility testing of clinical isolates is usually performed by disk diffusion or broth microdilution tests in diagnostic laboratories (Quainoo et al., 2017). WGS is a promising alternative. Analysis of WGS data for the presence of antimicrobial resistance genes and chromosomal mutations permits the prediction of antimicrobial resistance phenotypes, which can be >95% concordant with phenotypic testing for most of the antimicrobials tested in *E. coli* (Jiang et al., 2019). Nevertheless, WGS does not entirely replace phenotypic testing due to its high costs and possible emergence of novel antimicrobial resistance genes and mutations. Also, the relationship between phenotypic resistance and presence of resistance genes and mutations is complex. For instance, certain genes are responsible for resistance to several antimicrobials, and certain AMR genes and mutations are not sufficient alone to result in phenotypic resistance.

As the pathogenicity of ExPEC involves a large number of virulence genes that vary greatly between strains, WGS is ideally suited to more accurately predict role of an isolate in disease than a PCR for the detection of a limited number of virulence genes. For example, comparative genomic analysis of an ExPEC isolate from swine lungs provided strong evidence for a role of this strain as a causative agent in the pneumonia (Kong et al., 2017).

WGS allows improved infection control

In cases where vaccination with commercially available vaccines is not effective, as in the case of emergence of strains with new pathogenic properties, or when vaccines are not available, as in the case of ExPEC, characterization of the associated isolates by WGS permits the selection of the most appropriate strains for possible inclusion in an autogenous vaccine. We currently use this approach at the EcL. This characterization may identify new or variant adhesins important in the pathogenesis of diarrhea or septicemia (Fairbrother & Nadeau, 2019). Also, genomic analysis by WGS has been used to examine the effect of administration of autogenous *E. coli* vaccines on genetic diversity of *E. coli* isolated in poultry flocks (Lozica et al., 2021).

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Conclusions

Although the cost of carrying out the WGS for pathogenic *E. coli* remains relatively high, the comprehensive characterization of the entire genome and the high resolution in discrimination of related isolates makes it a very promising approach for use as a diagnostic test. This would translate into direct benefits for the veterinarian, either to replace existing techniques or to provide initial characterization of a smaller number of isolates to allow the setting up of targeted PCR for more large scale screening of isolates or samples.

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Struggling to control *Streptococcus suis* disease in the context of antibiotic reduction

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Introduction

Streptococcus suis (*S. suis*) is a major porcine pathogen responsible for important economic losses to the swine industry. In fact, it is one of the main causes of bacterial death in post-weaned piglets, from 5 to 10 weeks of age. According to the Canadian Swine Health Information Network, *S. suis*-related diseases are the most common infectious problem reported in Canadian swine farms. In addition, the Monitoring and Analysis Working Group from the Swine Health Information Center (SHIC) reviewed and established final rankings for what is now the Swine Bacteria Disease Matrix. As stated on its website, *S. suis* leads the list as the most important bacterial swine pathogen (https://www.swinehealth.org/swine-bacterial-disease-matrix/). Clinical features of these infections in pigs are meningitis, arthritis, endocarditis, polyserositis and septicemia with sudden death. The implication of *S. suis* as a primary respiratory pathogen has been seriously questioned, and it is now considered as a secondary agent of pneumonia. Isolation from lungs at slaughter should be considered as meanless and should not be taken into consideration. Outbreaks of *S. suis* disease result in decreased performance and increased mortality, which have a significant economic impact.

It is also a zoonotic agent and the infection in humans has attracted a high level of attention in last years, with deadly outbreaks reported in Asian countries, such as China and Thailand. There are at least 35 serotypes, based on capsular polysaccharide (CPS) antigens or CPS-related genes, although some of them have been proposed as being part of different bacterial species. Indeed, serotypes 20, 22 and 26 have been re-classified as *Streptococcus parasuis*, serotypes 32 and 34 as *Streptococcus orisratti* and serotype 33 as *Streptococcus ruminantium*. Previously described *S. suis* serotypes 20, 22, 26, 32 and 34 are still recovered from diseased pigs and many laboratories do still identify such isolates as *S. suis*. Sertoype 33 reference strain was originally isolated from an ill lamb (arthritis). So far, there is no single strain (confirmed by PCR) of this serotype recovered from swine: the few field strains reported in the past were identified by coagglutination (serological assay), a technique that presents many cross-reaction. When tested by PCR, these field strains were confirmed as being untypable or autoagglutinating. On the other hand, *Streptococcus ruminantium* (some of them detected as *S. suis* serotype 33 and others not) are frequently recovered from diseased ruminants (bovine and ovine) suffering from respiratory disease, abscess, arthritis, mastitis and other types of infections. *S. ruminantium* (and previous *S. suis* serotype 33) should not necessarily be considered as a porcine pathogen.

Among the serotypes described, type 2 is the most virulent and frequently isolated from both diseased pigs and humans. Serotype 9 is another important serotype involved in swine diseases However, this is the reality of Europe, which may be different in America, especially Canada and USA (Table 1). Phenotypically and genotypically different strains of *S. suis* serotype 2 (with different degree of virulence) have been isolated in different parts of the world. *S. suis* strains have also been analyzed and classified into clonal complexes (CC) composed by different sequence types (ST) when analyzed by multilocus sequence typing and, more recently, by whole genome sequencing, confirming the genetic heterogeneity within the *S. suis* species. Finally, untypable strains are also sometimes isolated from diseased animals and their possible virulence capacity should not be disregarded.

Pigs may acquire *S. suis* from the sows (during farrowing and also through oral/nasal contact) and through piglet-to-piglet transmission. Bacteria are localized in the tonsils, but they are also present in saliva: most pigs are carrier animals, harboring mostly low virulent strains. In the presence of virulent strains, some carrier piglets will eventually develop septicemia, meningitis and/or arthritis due to dissemination of *S. suis*, when maternal antibodies decline (between 5 and 9 weeks of age). The incidence of the disease is usually kept under 5% in the field. However, in the absence of prophylactic, methaphylactic and/or curative antibiotic treatments, mortality may reach 20%. Indeed, a significant increase of *S. suis*-related disease in post-weaned piglets has been observed in the last years, mainly associated to the reduction in the use of antibiotics. Although studies on *S. suis* have been significantly increased in the last 15 years, there are still many unresolved questions.

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Table 1: Distribution of different serotypes of *Streptococcus suis* (n=680) recovered from diseased pigs in Canada from 2015 to June 30, 2020.

Serotype	% ([a])	Serotype	% ([a])
1	5 (4)	18	<1 (1)
1/2	14 (10.5)	19	<1 (1)
2	12.5 (13.5)	20[b]	0 (0)
3	7 (7)	21	<1 (1)
4	6 (7)	22[b]	1 (4.5)
5	7 (2)	23	2 (3)
6	0 (<1)	24	<1 (0)
7	8 (6)	25	<1 (<1)
8	4 (5)	26	0 (0)
9	5 (4)	27	<1 (<1)
10	1 (<1)	28	<1 (<1)
11	<1 (<1)	29	1 (0)
12	<1 (<1)	30	<1 (1)
13	0 (<1)	31	3 (<1)
14	5 (2.5)	32[b]	<1 (<1)
15	<1 (<1)	33[b]	0 (<1)
16	2 (1.5)	34[b]	1.5 (<1)
17	<1 (0)	UT[c]	7 (18.5)

[a]Previous data from 2012 to 2014
[b]*S. suis*-like serotypes
[c]UT: untypable "real" *S. suis*

S. suis invades through the respiratory tract, gastrointestinal tract or both?

An overview of the pathogenesis of the infection can be observed in Figure 1.
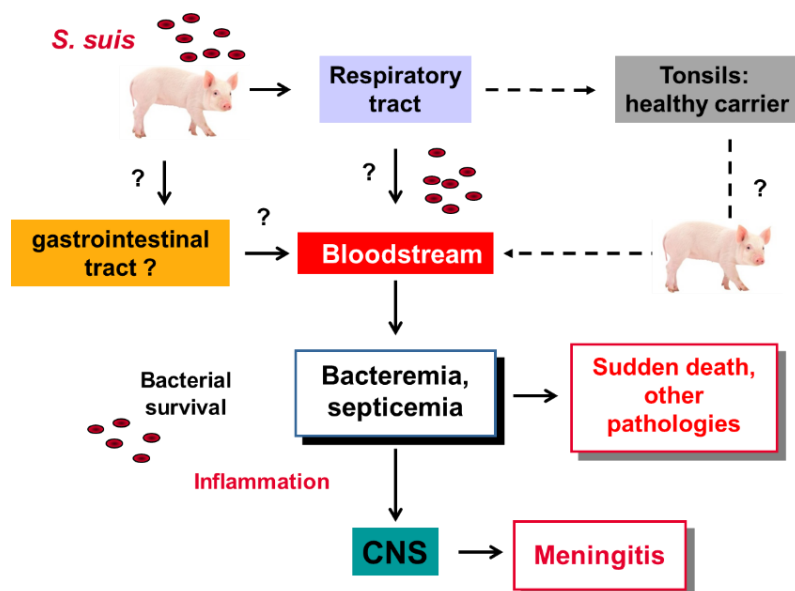
Figure 1: Proposed pathogenesis of the infection caused by *Streptococcus suis*

It has been always accepted (and demonstrated) that *S. suis* enter through the respiratory tract. Bacteria are then located at tonsils and remain there, being *S. suis* a normal inhabitant of the upper respiratory tract. Under stressful conditions and in the absence of antibodies (post-weaned piglets), potentially virulent strains invade the bloodstream (by still unconfirmed mechanisms) and induce bacteremia, septicemia and clinical signs (depending the colonized organs), through the induction of high levels of pro-inflammatory mediators. Sometimes, concentration of such mediators is so high that sudden death occurs and animals are simply found dead.

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More recently, a new route of entry has been proposed: the oral route, as it happens very often in humans (South-East Asia). However, this still needs to be confirmed. Studies reproducing disease mimicking this route of infection (for example: intestinal translocation) used either direct inoculation of high concentration of virulent *S. suis* in the jejunum or high concentration of bacteria included inside acid-resistant capsules and then given orally to pigs. Even under these circumstances, a very low number of infected animals developed disease. When post-weaned animals were challenged trough the oral route (without acid-resistant capsules), animals remained healthy. So far, many studies showing presence of *S. suis* in the intestine used DNA detection, so it is not easy to differentiate between live and dead bacteria. In addition, many of these studies included the use of genes that are not able to differentiate between *S.suis* and *S. suis*-like microorganisms. However, other studies isolated *S. suis* from feces and mesenteric lymph nodes. Indeed, in some cases of septicemia, animals may present diarrhea and *S. suis* (the strain responsible for the disease) can be isolated from feces and mesothelial nodes. However, it is not clear if the simply presence of virulent strains in the intestine of clinically healthy pigs is enough to induce disease. It is important to note that stress due to weaning and feed changes may significantly modify the intestinal environment causing stress to animals, which may be much susceptible to develop disease. Usually, first animals to develop disease are those in great shape and the biggest ones. Poor adaptation to solid feed may be even more important in such animals. Stress may induce invasion of *S. suis* into the bloodstream, but this may also happen from tonsils. More studies about possible pathogenesis of the infection through the gastrointestinal tract are needed before proposing new food additives to control *S. suis* infections (see below).

Definition of virulent strain of *S. suis*: a puzzle…

The majority of porcine *S. suis* infections are caused by strains belonging to a relatively small number of serotypes, especially in Europe/Asia. Although the distribution of serotypes from clinical cases differs depending on the geographic location, serotype 2 strains are responsible for the majority of cases in both swine and humans worldwide, and thus this serotype has been historically considered the most frequent and virulent type. However, this is true for Europe and Asia. In addition, serotypes 1 and 14 have also been described as highly virulent. Indeed, it has been shown that highly virulent strains of serotypes 1, 2 and 14 in Western countries are mostly included in CC1 (mostly ST1). These strains possess some virulence markers such as the suilysin (SLY), the muramidase-released protein (MRP) and the extracellular protein factor (EF), and presence of genes coding for such proteins can be detected by PCR and this is used in routine diagnosis in some laboratories. Besides this serotype, recent years have seen the emergence of serotype 9 strains among swine diseases in several European countries. Most of these strains isolated from diseased pigs (and differently from those recovered from tonsils of healthy pigs) belong to specific STs (such as ST16, ST123 and ST125) and produce SLY and a variant of the protein MRP. Interestingly, it is not easy to reproduce disease with such "virulent" serotype 9 strains and usually highly aggressive intravenous or intratracheal infections are needed. Intranasal infection does not usually induce disease, even with high susceptible caesarian derived colostrum deprived piglets.

What happens with strains isolated in America? One striking observation is that the percentage of *S. suis* serotype 2 strains recovered from diseased pigs is lower in North America than in other parts of the world. In addition, human *S. suis* disease cases are rarely reported in Canada and USA. In fact, it has been shown that serotype 2 strains in North America are less virulent and genetically unrelated to those causing disease in other parts of the world, such as Europe and Asia. Serotype 9 strains are also different and belong to a bunch of different STs, not usually associated with disease. Highly virulent serotypes 1 and 14 (CC1, ST1) are, however, present in these two countries. On the other hand, most isolates from diseased pigs belong to different serotypes (such as serotype 1/2, the most prevalent in USA and Canada) and most serotype 2 isolates belong to CCs different from CC1 (Table 2). Analysis of virulence markers (MRP, EF and SLY) does not give any additional information since most serotype 2 strains are negative for SLY and EF and serotype 9 strains are negative for SLY. On the other hand, most of the North American serotypes 1 and 13 isolates do belong to the CC1 group and are SLY, MRP and EF positive. In Latin America, there is not much information and the presence of highly or lower virulent strains of serotype 2 may depend on the source (geographical region) of genetics. Data from Argentina (a country with more of 20 human cases described, the highest number of cases in whole America) indicate that highly virulent serotype 2 CC1 (ST1) strains are widely present, similar to what is observed in Europe. Data from Brazil, the most important swine producer in Latin America, are missing. In addition, no human cases has been reported, which may indicate a problem of misidentification in laboratories of human medicine.

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Table 2: Distribution of sequence types and respective clonal complexes of *Streptococcus suis* serotypes 1, 2 and 14 isolates from recovered from Canada between January 2015 and June 2020 (n = 146).

Serotype (No. isolates)	Sequence type (No. isolates)	Clonal complex
1 (35)	1 (27)	1
	13 (1)	13
	28 (1)	28
	94 (6)	94
2 (78)	25 (30)	25
	28 (35)	28
	620 (11)	
	1617 (2)	Unknown
14 (33)	1 (16)	1
	13 (1)	13
	19 (1)	87
	28 (3)	28
	94 (9)	94
	839 (2)	
	1616 (1)	Unknown

Do *S. suis*-related diseases depend on the presence of virulent strains only?

S. suis-associated diseases are complex. The presence of a potential virulent strain alone does not guarantee appearance of clinical signs and, sometimes, clinical signs are observed in the absence of such strains. Virulence of strains belonging serotypes other than serotypes 1, 2, 9 and 14 is almost unknown, there are no virulence markers and there is no validated model of infection in pigs, since in most cases, animals infected with strains belonging to other serotypes will not develop disease. However, these strains are commonly found in *S. suis*-associated diseases, especially in North America. This is still one of the major challenges we face in the diagnosis of *S. suis* infections: how to determine if a given strain (from non-serotypes 1, 2, 9 or 14) isolated from a diseased animal is, in fact, really responsible for most clinical cases in a farm. We usually recommend performing a necropsy of at least 3 animals from the same batch, in 3 different batches. If *S. suis* is isolated in pure or predominant culture from internal organs (other than lungs) in most of these animals, and serotyping indicates that one or a very few serotypes are always involved, we can conclude that those strains are probably important. If inconclusive results are obtained, additional animals should be analyzed: if at the end, 4-5 or more serotypes are detected, predisposal factors should be taken into consideration before planning the use of an autogenous vaccine (see below). The analysis of the results is even more complicated if untypable strains are involved: are these all the same strain? Or different untypable strains are causing disease? These questions can only be answered through the sequencing and comparison of such strains.

> Although many *S. suis* strains have been studied by whole genomic sequencing (WGS) and different virulence-related genes have been proposed as virulence markers, none of them could be so far validated in the field, at least with North American strains (upublished data). Up to know, there are no validated markers to clearly identify all pathogenic strains and information obtained by WGS, although interesting from the epidemiological point of view has limited application.

In fact, when multiple strains are involved, other factors may influence the presence of disease (Figure 2). However, *S. suis* is also the disease of the exceptions, since everything may happen. In rare conditions, 3-4 different virulent strains affecting animals from the same herd (example: serotypes 1, 2 and 14, all ST1, or a serotype 2 ST1 and a serotype 9 ST16) may also occur and this complicate even more the diagnosis. We have recently observed animals dying at the same time with pure culture isolation of a serotype 2 (one animal) and a serotype 14 (the second animal), both virulent strains.

Obviously, the presence of virulent strains is an important factor. However, concomitant infections with other pathogens may highly contribute to the presence of clinical signs. The most important, by far, is the instability of the farm to Porcine Reproductive and Respiratory Syndrome (PRRS) virus. Although data from the field clearly show that a PRRSv previous infection predisposes to clinical disease caused by *S. suis*, the exact mechanisms involved are still unknown. It has been also shown that previous infection with Aujeszky virus may predispose animals to an enhanced *S. suis* disease. Swine influenza virus (SIV) may also enhances the infections caused by *S. suis,* especially with strains that possess sialic acid in their capsule: serotype 2 but also serotypes 1, 1/2 and 14. Unfortunately, there

are no scientific data concerning other co-infections, such as those caused by porcine circovirus, mycoplasma or others. Co-infections (with clinical disease) with *Glaesserella parasuis* seem to be rare. The presence of mycotoxins has also been suggested as a predisposing factor but it has never been scientifically studied (studies are presently ongoing in our laboratory).

It is also considered that environmental factors may also greatly influence the appearance of *S. suis*-related diseases, such as (among others) poor ventilation, high humidity, inadequate sanitation and important temperature variation between night and day. It is interesting to comment here how experimental infections by the intranasal route are done with virulent strains. If a high concentration of a virulent strain of *S. suis* serotype 2 is inoculated to the nasal cavities of conventional pigs, usually few or no clinical signs are observed. A previous treatment done with acetic acid to irritate the nasal mucosa should be done, followed by the infection with the virulent strain. Under these conditions, clinical signs may be observed, and this will depend on the farm from where the animals originated. This indicates that other environmental factors, such as high levels of dust and ammonia (causing irritation) or co-infections (toxigenic strains of *Bordetella bronchiseptica* and/or *Pasteurella multocida*) may greatly influence animal susceptibility to develop clinical signs. Chances to reproduce disease will considerably increase if some kind of stress is applied to the infected animals. Although *S. suis* may be clearly a primary etiological agent of disease, **the Koch's postulates** sometimes **are not easy to be reproduced** with this difficult pathogen.

Management factors may also influence the development of *S. suis*-related diseases. For example, high level of cross fostering, overcrowding, teeth clipping and tail docking (arthritis), ear notching (arthritis), mixing pigs of different ages, poor adaptation to solid feed in the nursery and low levels of vitamin E. It has been also suggested that the use of strong antibiotics given at the first week of life may been associated with the increased presence of clinical signs due to *S. suis*. Influence of the weaning age (ex: 3 vs 4 weeks of age) has not been studied yet.

Finally, our studies have demonstrated that the immunological status of animals is extremely important. Indeed, most clinical cases occur when maternal antibodies declined (between 5 and 9 weeks of age) and before natural antibodies are produced. Indeed, natural antibodies slowly increase from 9-10 weeks of age: nobody knows if such antibodies are directed specifically against *S. suis* or to other antigenically-related bacteria present in tonsils. These antibodies are always present in pigs (even in the absence of *S. suis*-related clinical disease in post-weaned animals), increase with age and are probably protective. This may explain why clinical cases are rarely observed in adult animals.
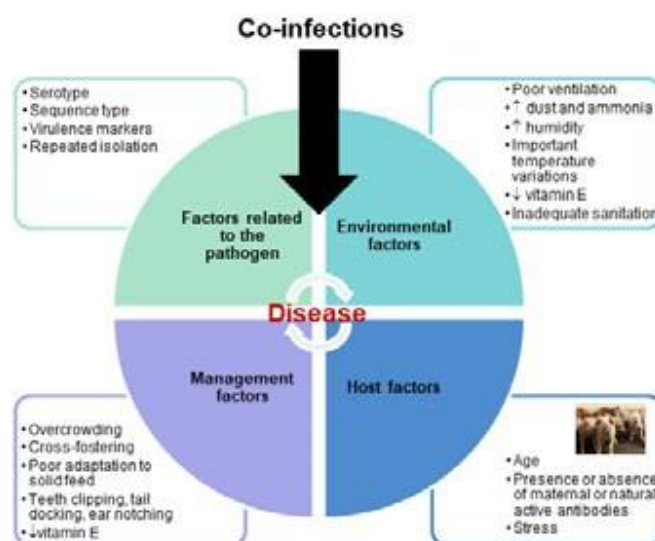

Figure 2: Factors contributing to the expression of *S. suis*-related diseases

Does *S. suis* represent a danger for antibiotic resistance?

As mentioned before, the incidence of the disease is usually kept under 5% the field, but this is mainly due to the extensive and routine (where allowed) prophylactic and metaphylactic use of antibiotics. In some countries, where antibiotics in feed are banished, these are sometimes used in water. The problem of the antibiotic use for *S. suis* infections is not necessarily the development of resistance of such strains to the most frequently molecules used to treat *S. suis*-affected pigs. The antibiotics of choice for this type of infection are beta-lactams. Most strains able to cause disease are susceptible to such drugs and this is due to the mechanism of resistance: it is not mediated by enzymatic degradation of the beta-lactam molecules (beta-lactamases), but rather involves modifications in the form of altered molecular weight and/or a decrease in the penicillin-binding capacity of its beta-lactam target proteins: the penicillin-binding proteins (PBP). Indeed, an increase in resistance to penicillin and amoxicillin by *S. suis* is brought

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by distinct cumulative alterations in its PBPs which happen at the chromosomal level. The consequence of such mechanism of resistance is that it may take years to develop some kind of resistance and, if it develops, it progresses very slowly. On the other hand, it is important to keep testing field strains to confirm such a hypothesis.

It is important to note that some diagnostic laboratories do not use standardized methods to measure resistance, so equivocal results are sometimes observed, as for example: sensitivity to penicillin but resistance to amoxicillin or resistance to amoxicillin but susceptibility to amoxicillin with clavulanic acid: this is simply unlikely to be true. If some kind of resistance to beta-lactams is observed, it is recommended to send the isolate to an independent laboratory to repeat the test. Some strains isolated from tonsils may present lower level of susceptibility, event to beta-lactam antibiotics: most of these strains are non-encapsulated, usually non-typable and probably non-virulent. In addition, many of these bacteria do not even belong to the *Streptococcus suis* species.

So, can we say that antibiotic resistance is not important for *S. suis*? It should be remembered that some of the antibiotics used are also of importance for human medicine. In addition, worldwide data from resistance of *S. suis* to antibiotics are alarming. Field strains are highly resistant to many different antimicrobials (such as tetracyclines and erythromycin), even if they are still susceptible to beta-lactams. Indeed, *S. suis* is considered **a niche for antibiotic resistance** and represents **a high risk of transmission of resistance** to other pathogens. This arises from **mobile genetic elements** in *S. suis* carrying resistance genes that are transferable at high frequency not only between *S. suis* strains but also **to other bacterial species**. Again, it is important to emphasize the need for continuous surveillance of resistance patterns in all pig pathogenic bacteria.

How to prevent disease caused by *S. suis* without using antibiotics?

Restrictions in the use of antibiotics brought, among other consequences, an increase of clinical disease in post-weaned piglets. In farms where animals are raised without antibiotics, *S. suis* is one of the most important concern. The question everybody is asking: how can we prevent *S. suis* diseases? Everybody also agrees that controlling stress and predisposing factors (concomitant infections, environmental and management factors, etc.) may significantly help to reduce disease. However, this is frequently not enough. What else can be done?

There are many alternatives to antibiotics that have been tested for *S. suis*. However, most of them have been tested *in vitro* but not *in vivo* (Table 3). So far, very few compounds have been clearly demonstrated as being effective to control *S. suis* disease *in vivo*.

Table 3: Different products tested as an alternative to antibiotics to kill *S. suis*

Product	Tested	Effect observed	Publication (peer reviewed)
Probiotics	*In vitro*	Yes	Yes
Phages	*In vivo (mice)*	Yes	Yes
Defensins	*In vitro*	Yes	Yes
Bacteriocins	*In vitro*	Yes	Yes
Galabiose	*In vivo*	No	Yes

The use of feed additives became also popular, based on the hypothesis of *S. suis* causing disease through the intestinal route, as discussed above. However, some available studies either have been tested *in vitro* or have not been published in peer-reviewed journals and lack the strict evaluation from the scientific community. Some examples are present in Table 4. Again, the use of these products become more and more popular, but more scientific data indicating any advantage to use them to control and prevent *S. suis* infections are needed.

Table 4: Feed additives tested to control *S. suis* infections

Product	Tested	Publication (peer reviewed)
Lauric acid	*In vitro*	Yes
Lauric acid	*In vivo*	No
Fatty acids	*In vitro*	No
Fatty acids + Lysozyme	*In vivo*	Yes
Cinnamon, oregano, thyme	*In vitro*	Yes
Basil, rosemary, peppermint	*In vitro*	Yes
Cello-oligosaccharides	*In vivo*	No
Alfalfa	*In vivo*	No

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As the complexity of *S. suis* epidemiology in swine increases (multiple strains, multiple serotypes), field reports describing difficulty in disease control and management are common. A logical alternative is the use of vaccines. However, so far, there is no commercial vaccine able to protect against all serotypes/strains of *S. suis*. Many research studies evaluated sub-unit vaccines (proteins) or even live vaccines, but controversial results have been obtained. The consequence is that the only alternative practitioners have in hands is the use of bacterins (killed whole bacteria), mostly autogenous vaccines. Autogenous vaccines are bacterins based on the predominant strain(s) recovered from diseased pigs in the affected farm and produced by accredited laboratories. Most published studies have been done with bacterins produced in research laboratories with reference strains, a kind of artificial "autogenous vaccine", not produced by accredited laboratories.

For the production of an autogenous vaccine, the first step is the choice of the strain. Differently from *Glaesserella parasuis*, *S. suis* is easy to isolate. However, under certain circumstances, different strains may be isolated from diseased pigs within the same farm (see above4). *S. suis* may be either a secondary or primary pathogen: as mentioned, co-infections, environmental or management issues may help moderately virulent *S. suis* strains, normally located in tonsils, to induce disease. In these cases, it is better to concentrate the efforts to reduce predisposing factors, as *S. suis* disease is a consequence not really a cause of the health problem. Diagnosis of *S. suis* infection as primary pathogen is not easy. Figure 3 shows a standard procedure that may help on the decision to incorporate a given strain to an autogenous vaccine.
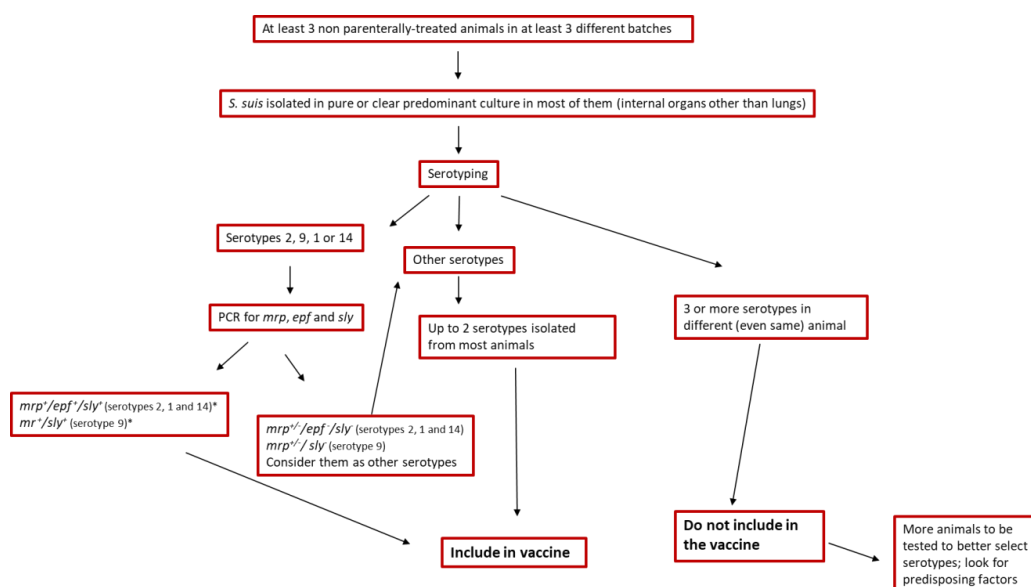

Figure 3: Proposed methodology to choose strains to be included in an autogenous vaccine. *For Europeanstrains only.

In some farms, autogenous vaccines include 4-5 serotypes of *S. suis* and sometimes other bacterial species, such as *Staphylococcus hyicus*, *Streptococcus dysgalactiae*, *Glaesserella parasuis*, *Erysipelothrix rhusiopathiae* and/or *Actinobacillus suis*. Although never studied, the inclusion of such huge mass of antigens may have two different consequences: a) the reduction of the bacterial concentration of each individual strain to keep a 2 ml vaccine, and/or b) distraction of the immune system. It is hard to evaluate if all strains are necessary and if the immune system is able to produce antibodies to all relevant antigens. Finally, the production of autogenous vaccines is more an "art" than a "science". The value of autogenous vaccines, at large, cannot be evaluated. Why? Because each company produces the vaccine differently, and most of the variables have never been studied. Some of the variations that may happen among different vaccine productions are presented in Table 5.

Table 5: Some variables that may be present when producing an autogenous vaccine

Characteristic	Variables
Bacterial growth (a)	Exponential vs Stationary
Bacterial growth (b)	Solid medium vs Liquid medium
Bacterial growth (c)	If liquid medium: shaking flasks? biofermentor?
Bacterial growth (d)	Type of medium
Bacterial growth (e)	Aerobic, microaerophilic or anaerobic conditions
Body of the vaccine	Washed bacteria or bacteria + supernatant
Bacterial concentration	High vs Very high; keep concentration when several serotypes included?
Inactivation procedure	Formalin vs others
Adjuvants (a)	Type of adjuvant: type of immune response?
Adjuvants (b)	Concentration?

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Indeed, under each condition of vaccine production, different antigens may be expressed. In addition, it has also been demonstrated that adjuvants used may greatly influence the protection observed with experimental vaccines. So, it is impossible to compare autogenous vaccines produced by different companies. This explains in part why there are almost no scientific data evaluating autogenous vaccines in general in the field, at least published by peer-reviewed journals. Most data are from internet or oral presentations in different congress and other meetings. In addition, most studies do not include control groups: the contribution of the autogenous vaccine to the control of the infection is

normally evaluated by studies done "before" vs "after", where mortality and the use of antibiotics to treat animals are compared. One of the problems is that, normally, "mortality" refers to total mortality in the nursery…not necessarily mortality related to *S. suis* only. In addition, other measures to control predisposing factors may also be applied simultaneously with vaccination, which may complicate the analysis. On the other hand, the inclusion of a control group may not solve the problem: when mortality is under 5%, a significant high number of animals must be included in both groups, since otherwise it is difficult to observe differences. Finally, there are no studies where the antibody response of vaccinated animals with a commercial autogenous vaccine have been evaluated, so it is unknown if such vaccines are able, at least, to induce an increase of the antibody levels, which would be an indirect way to study potential protection.

As mentioned, adjuvants can dramatically influence the vaccine-induced antibody response, as it was studied with experimental (noncommercial) vaccines. Not all antibodies (immunoglobulins or IgG) induced by a bacterin are indeed protective. Some IgG subclasses (called "isotypes"), such IgG2 and to a lesser extent IgG3, are particularly effective at mediating bacterial ingestion and destruction by leukocytes (phagocytosis). Indeed, *S. suis* resistance to phagocytosis and thus innate immunity clearance, is lost in the presence of antibodies that promote bacterial phagocytosis and destruction by professional phagocytes (called "opsonophagocytosis" or OPA test). Other antibodies isotypes, such as IgG1, even if they are induced after vaccination and recognize the pathogen, are much less protective, since they cannot help the host to destroy the pathogen. Interestingly, different adjuvants may influence the production of "protective" or "non-protective" IgGs. An *in vitro* OPA test has been lately standardized to measure protective activity of antibodies against *S. suis*, although it has never been used so far to measure the antibody response against field autogenous vaccines. So, not only levels of antibodies should be measured, but also their functionality. We are presently developing such tests in our laboratory and results will be available soon.

Finally, there are no clear data when and how autogenous vaccines should be applied, and there are almost no scientific studies available. In the field, and without any scientific data, autogenous vaccines are used either in sows (mostly) or piglets or (less common) both. Vaccination of sows before farrowing might elicit passive maternal immunity, being less costly, and thus representing an economical alternative to piglet vaccination. Yet, available results indicate that pre-farrowing immunization of sows with these experimental bacterins to protect piglets gave inconclusive results with either no antibody production, antibody production that did not protect piglets or a restricted protection for piglets of less than 6 weeks of age. In fact, piglets at late nursery remain without antibodies, a period of high risk of *S. suis* disease in many farms. Active vaccination of young animals, such as suckling piglets, has the concern of possible interference with maternal antibodies. Indeed, neither vaccination of suckling nor of weaning piglets from immunized sows with experimental bacterins was constantly associated with a prominent active immune response and protection at 8 weeks of age. In this regard, interference between maternal antibodies and active production of antibodies against *S. suis* was also suggested in a field study with also an experimental bacterin. Vaccination of older piglets (for example, at 3 and 5 weeks of age) may not induce a booster antibody production early enough to protect piglets at the nursery: there is clearly a problem of a window of vaccination. Knowledge of antibody kinetics is thus required before implementation of a rational vaccination program. The adopted strategy should allow minimal interference between passive maternal immunity and active immunization in piglets but maximal protection for pigs at the approximate time of onset of clinical signs.

Conclusions

S. suis infections are multifactorial and very difficult to control. With the reduction in the use of antibiotics, nursery mortality due to this pathogen significantly increased. *S. suis* may be a primary or secondary pathogen and control of predisposing factors should not be neglected. Evaluation of the virulence of the involved strains is not easy to perform under all circumstances; in addition, the virulence potential of the strain is only one of the aspects to be taken into consideration to control disease. Products that can used as alternatives to antibiotics need still to be scientifically evaluated. Finally, due to limited field reports concerning immunogenicity and protection, the usefulness of autogenous bacterins in a rational vaccination program remains to be proved and needs further research. Studies where independent researchers evaluate the influence of different aspects of the production of an autogenous vaccine on their protective capacity are also required. As it is still the only option available to be used as immunogens, a diagnostic effort on the evaluation of the strains to be included in such vaccine must be done. Finally, autogenous vaccines must be produced by companies having a large experience on this field.

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The path ahead for Mycoplasma control

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Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) continues to be a significant pathogen for pigs worldwide. The bacterium is responsible for respiratory infections, which can get complicated with other agents and lead to the development poly-microbial diseases like the Porcine Respiratory Disease Complex. Mycoplasma infections are characterized by being endemic and slow in nature. Pigs in affected herds mainly exhibit high susceptibility to infection with other pathogens and long time to reach market weight. During more than half of a century, practitioners and producers have dealt with *M. hyopneumoniae* and the disease it causes in a number of different ways, generally achieving partial control. However, total control can only be accomplished when the elimination of the agent, and thus the disease, are targeted. In this summary, a revision of the main strategies for disease control is presented, along with a call for increased efforts in the disease eradication arena at large scale.

The focus of control programs has changed

A common complaint heard among swine practitioners in recent years was the lack of disease control when employing traditional control methods. In other words, practices that offered tangible results when attempting to decrease the effects of *M. hyopneumoniae* infections did not seem to work the same way they did in the past. A revision of current inputs, practices and epidemiological information lead to identify that herd destabilization was driven by the way in which sow farms were managed. Infections caused by *M. hyopneumoniae* were thought to be diseases of growing pigs only, and attention to them in sow farms was minimum. A significant shift has occurred in how *M. hyopneumoniae* infections are perceived at the sow farm and today, control programs that do not involve working on the sow farm are rare. It is very well understood that due to the slow nature of the pathogen, infections start as early as the lactation period, and growing pigs carry the agent into nurseries and finishers, becoming amplifiers for the entire group. At the sow farm, it is now common knowledge that shedding females are the single most important factor determining young piglet colonization. Therefore, efforts for disease control should start even before a litter is born. In fact, control efforts should start prior to gilts entering the sow farm. Thus, a new focus for Mycoplasma disease control has been established.

Current strategies for control of *M. hyopneumoniae* infections

Nowadays, sound Mycoplasma control programs that are not directed at pathogen elimination (eradication) usually include a combination of the following three key strategies: Management, prevention and treatment. Although each strategy is quite different in nature, they work synergistically most times, and lead to the improvement of swine health and production, achieving partial disease control. On the other hand, the ultimate form of disease control for *M. hyopneumoniae* infections is pathogen eradication. Following is a brief description of the main aspects surrounding each one of the strategies for disease control:

- **Optimization of management practices**. The first aspect to revise when attempting to control respiratory disease is herd management. This is a very broad aspect in porcine production, as multiple factors will play a role on the final outcome, but mainly include: the type of production system, climate and housing conditions, animal introductions to the herd, etc. The *type of production system* dictates how pigs from different ages and production stages will flow within the system, and with that, the potential for personnel movement, equipment sharing, contact between pigs, and disease transmission. These days, the swine industry relies in multi-site pig production to avoid contact between pigs of different ages and this has allowed to minimize disease expression, even in infected herds. The *climate and housing conditions* include facilities, ventilation, stocking density, climate, among others that will have a significant influence on the development and transmission of diseases affecting the respiratory tract. Adequate and well calculated design of facilities based on climatic conditions and neighboring populations is of paramount importance. Controlled *introduction of incoming gilts* to endemically infected sow farms has become one of the single most important practices to control *M. hyopneumoniae* in large farms in recent years. Gilt acclimation including *M. hyopneumoniae* controlled exposure can be applied for disease elimination or to achieve stabilization in herds when pig populations with opposite health statuses are to meet.

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- **Vaccination**. For decades, bacterins to control *M. hyopneumoniae* have been commercially available. Preparations are mainly constituted of adjuvanted killed bacteria, which help decrease clinical signs associated with infection, improve production parameters, decrease lung lesions and have the potential to decrease pathogen transmission at the population level (especially when applying multiple doses), although they do not prevent infection. Vaccine products are usually administered to growing pigs prior to weaning, alone or in combination with other antigens. Replacement gilts are generally vaccinated as wean pigs and once they enter the sow farm. Vaccine preparations are very safe and have been updated over the years, mainly to allow more flexibility of administration and decrease labor. Thus two-dose vaccination schemes are rarely employed in growing pig populations for the most part.
- **Antibiotic treatment**. Being a bacterium, *M. hyopneumoniae* can be treated with antimicrobial compounds, although the lack of cell wall makes Mycoplasmas naturally resistant to certain classes of antibiotics, such as β-lactams. It is also important to keep into account several aspects when thinking about antimicrobial treatments and Mycoplasma infections. First of all, antimicrobial treatment will decrease the effect and extent of infection at the lung level, and decrease the potential for disease transmission among pigs, but treated individuals will not be completely clear from infection after antibiotic treatment.
- **Pathogen eradication**. The total elimination of a pathogen from a host population can seem an overwhelmingly and sometimes unrealistic task, and although certainly not easy to perform, *M. hyopneumoniae* infections have been and are being eliminated from swine farms all over the world, and only when successfully completing *M. hyopneumoniae* eradication programs, can total disease control be attained.

Total Mycoplasma control can be achieved in various ways

Examples of *M. hyopneumoniae* eradication programs have been documented for more than 30 years. Several strategies have been used to eradicate the agent and eliminate the disease it causes. Here, a short description of each of the most common ways to conduct Mycoplasma eradication programs is presented:

- Depopulation and repopulation: Maybe the most straight forward approach for disease elimination, depopulation and repopulation implies culling the entire herd, cleaning facilities and bringing in new pigs to a production unit. Although the concept is very clean cut and easy to understand, the implications from the genetic, production and cost stand points can be too large for implementation of this method in most cases. However, the potential to eliminate various agents in a single effort make this approach attractive in specific circumstances.
- Partial depopulation: Pioneering the Mycoplasma eradication area, partial depopulation, also known as the Swiss method, was applied in small farms in the late 1980s. This program is based on the premise that pigs are housed in a single facility, which ensures early exposure to the pathogen and transmission in the population. The program involves culling of all pigs younger than 10 months of age, skipping two weeks of farrowing and medicating the entire herd during the break in production. The high success rate of this program is public and its best example is the application at the national level in countries like Switzerland and Finland. Nevertheless the particularities of this program make it unsuitable for large and/or multi-site farms.
- Herd closure and medication: Designed on the basis of the partial depopulation program, described above, and the most used strategy to control porcine reproductive and respiratory virus (PRRSV) infections, herd closure and medication combines the segregation of pigs of certain ages, medication, and vaccination. The segregation of certain age groups, although long, is temporary, and is applied in order to avoid the transmission of the pathogen from already infected groups of pigs (sows) to naïve ones (in this case gilts and newborn piglets). Generally, herd closure is employed when PRRSV eliminations are targeted and imply the extension of the closure up to at least 240 days, when clearance of *M. hyopneumoniae* from infected pigs has been shown. Medication is administered to the entire herd at certain specific times and multiple vaccination is also practiced. The application of this method has grown in North America in recent years based on its success for disease elimination, especially when combined with early purposeful exposure to *M. hyopneumoniae* in gilts that are to enter the sow farm.
- Whole herd medication: Usually applied after a Mycoplasma disease outbreak, whole herd medication takes advantage of the natural exposure that occurs in a herd and is combined with a blanket medication using injectable antibiotics.

The success rate at eradicating *M. hyopneumoniae* varies depending on the method that is applied. For the most part, depopulation and repopulation exhibits the greatest success rate, when properly performed and when potential external pathogen sources do not exist, or do not pose a risk to the farm. Reports in the literature refer to approximately 80% success rate when using the Swiss method. Similarly herd closure and medication exhibits success rates that can reach almost 80%, while whole herd medication programs are documented to have between 50 and 60% success. However, even when eradication is not achieved, the benefits of reaching a very low prevalence of the agent are evident and highly desirable.

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The path ahead

The swine industry is characterized for being progressive, responsible and adaptable. Today's challenge is to keep producing high quality animal protein to feed a growing world population, without hurting the planet. As swine veterinarians, it is our role to lead food animal production under the strictest health and production standards, and disease elimination is a central piece of the sustainability plan that is entirely in our hands to apply and to move forward. Therefore, the invitation is to think big and bold, and to aim for pathogen eradication, at least for those agents that can be put behind us, like Mycoplasma. Swine veterinarians have employed clinical, diagnostic, prevention, and control tools in several different conditions and they have shown that it is possible to grow pigs and to maintain herds that are free of *M. hyopneumoniae* infections. So, what are we waiting for to lead the way?

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Interactions of the gut microbiome and respiratory viruses

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The Porcine Gut Microbiome

The gut microbiome is the term used to describe the diverse microorganisms that live throughout the length of the gastrointestinal tract, including bacteria, viruses, fungi, protozoa and archaea. Microbes in the gut are estimated to equal or outnumber cells within the host (Sender, Fuchs et al. 2016). At least three important roles of the gut microbiome are recognized in the context of host health and disease, including the provision of a protective intestinal barrier preventing pathogen binding, metabolism of complex carbohydrates and other nutrients, and development and maintenance of immunity. Within the first several weeks of a pig's life, there are numerous events which provide opportunities to beneficially or detrimentally impact gut microbiome colonization. Summarized in **Figure 1**, factors including maternal gut and vaginal microbiomes, mammary skin and milk microbiomes, piglet stress at processing or transport, injectable or in-feed antimicrobial administration, diet composition and weaning time, pathogen exposure and environmental conditions have all been shown to potentially impact the gut microbiome for the lifetime of a production pig (Niederwerder 2017).
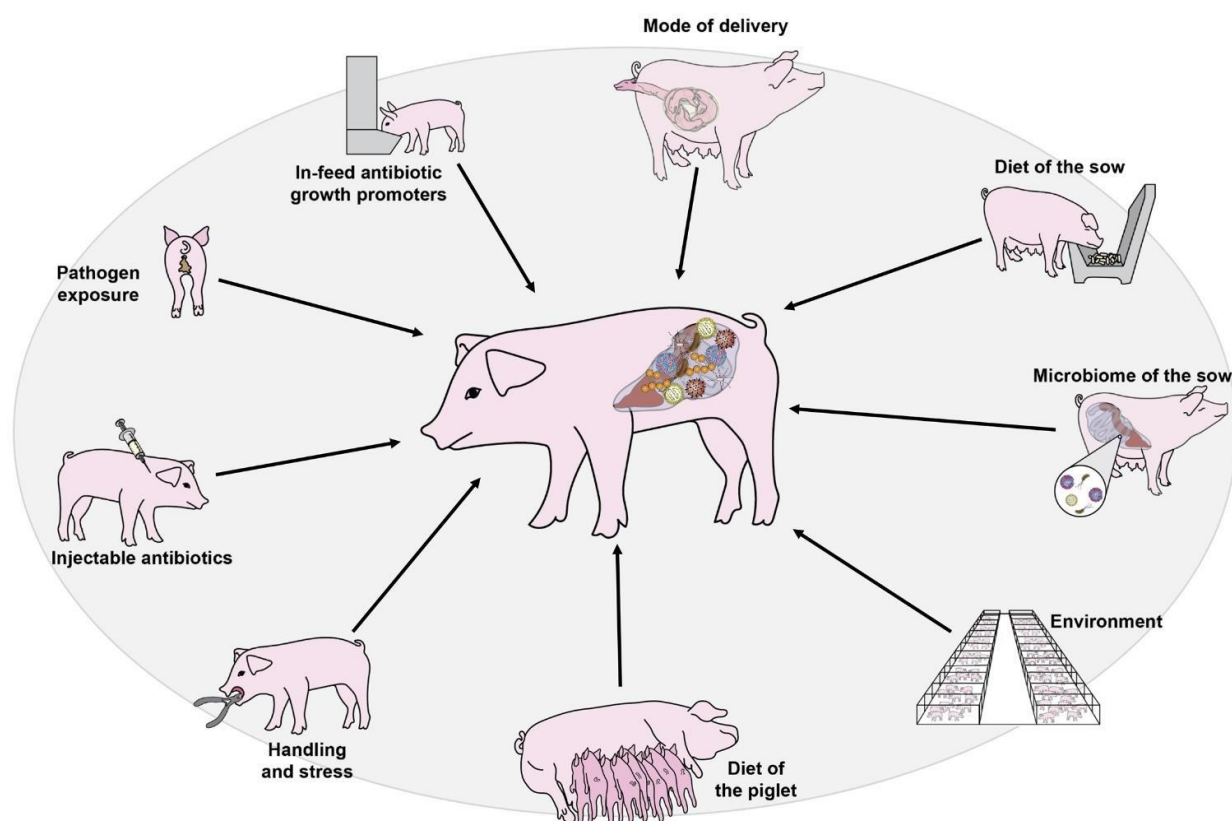

Figure 1. Potential factors which may beneficially or detrimentally impact gut microbiome colonization in the first several weeks of a production pig's life. Figure adapted from Niederwerder (2017).

Role of the Gut Microbiome in Disease

Over the last two decades, the gut microbiome has emerged as having significant associations with several disease states in both humans and animals. The relationship, balance and mechanistic interactions between the gut microbes and host disease states are complex and often poorly defined. However, there is growing evidence supporting the beneficial role that gut microbiome diversity and composition play in the outcome of pathogenic infections.

Specifically, gut microbiome characteristics, such as reduced diversity and shifts in phyla composition, have been associated with infectious disease outcomes both inside and outside the gut. When considering respiratory disease, the gut-lung axis is the term used to describe how the gut microbiome communicates with the respiratory system in a

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bidirectional manner. Communications are believed to occur through bacterial metabolic products, lymphocyte migration to distant sites, and inflammatory mediators after stimulation of mucosal immunity (Zhang, Li et al. 2020). Several associations have been characterized between the gut microbiome and severity of pneumonia after infection with fungal, viral and bacterial respiratory pathogens which affect humans (Niederwerder 2017, Rosshart, Vassallo et al. 2017, Grayson, Camarda et al. 2018, Antunes, Fachi et al. 2019, Zuo, Zhang et al. 2020).

Porcine Respiratory Disease Complex

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) are two of the most widespread and significant pathogens affecting swine worldwide, costing the pork industry billions of dollars in losses over the last 30 years. Both viruses cause systemic infections, resulting in primary pathology of lymphoid and pulmonary tissues, and are commonly detected in cases of polymicrobial respiratory disease. Infections with PRRSV and PCV2 result in several immunological outcomes, including pathology of macrophages and lymphocytes, causing immunosuppression and enhancing the susceptibility of growing pigs to primary and secondary pathogens. Although the currently available PRRS modified live virus (MLV) vaccines are widely used to reduce PRRS-associated production losses, they are generally considered inadequate for disease control and eradication (Nan, Wu et al. 2017, Vu, Pattnaik et al. 2017, Montaner-Tarbes, Del Portillo et al. 2019). Programs designed for long-term elimination of PRRSV from a herd are often unsuccessful and reintroduction of PRRSV is common. Polymicrobial respiratory infections are a significant challenge to swine production due to reduced weight gain, morbidities and mortalities, decreased animal welfare, and the need for increased antimicrobial administration. By utilizing the gut-lung axis, microbiome modulation and the use of beneficial gut bacteria is a potential alternative tool for reducing the effects of polymicrobial respiratory disease on growing swine.

Gut Microbes, PRRSV and PCV2

Our laboratory has focused on identifying associations between the gut microbiota and disease outcome in nursery pigs co-infected with PRRSV and PCV2. Initial proof-of-concept work demonstrated that characteristics of the gut microbiome were associated with clinical outcome of weaned pigs 70 days post-infection with PRRSV/PCV2 (Niederwerder, Jaing et al. 2016). Specifically, reduced clinical signs, improved weight gain, and reduced pulmonary pathology was associated with increased microbiome diversity and the presence of a nonpathogenic *Escherichia coli* in the gut. Follow up work determined that pre-infection gut microbiome characteristics were also associated with subsequent outcome after co-infection with PRRSV/PCV2 (Ober, Thissen et al. 2017). Overall, pigs with high growth rates, reduced virus replication, and less severe pneumonic lesions had several gut microbiome characteristics that may have predisposed outcome, including increased microbial diversity, reduced *Methanobacteriaceae* species, increased *Ruminococcaceae* species, and increased *Streptococcaceae* species. Further, we determined that pre-challenge gut microbiome characteristics were associated with growth outcome in pigs immunized with a PRRS MLV vaccine and subsequently challenged with PRRSV/PCV2 (Constance, Thissen et al. 2021). When compared to pigs with low growth rates, high growth rate pigs had several gut microbiome characteristics after vaccination that may have predisposed outcome to challenge, such as increased bacterial diversity, increased *Bacteroides pectinophilus*, and increased *Lachnospiraceae* species C6A11 and P6B14.

Fecal Microbiota Transplantation and Porcine Circovirus Associated Disease

Fecal microbiota transplantation (FMT) is the process by which feces is collected from a healthy donor and transplanted into a diseased or young individual to modulate the gut microbes. FMT not only transplants live and dead microbes, but also small feed particulate, cells from the small and large intestine, and metabolic products from microbes (Bojanova and Bordenstein 2016). The mechanism by which FMT is efficacious is believed to be due to an increase in favorable microbes, restoration of normal flora, an increase in microbial diversity, and stimulation of mucosal and systemic immunity (Niederwerder 2018).

Our laboratory investigated the use of FMT as a method to modulate the gut microbiome for the prevention of disease after co-infection with PRRSV/PCV2 (Niederwerder, Constance et al. 2018). **Figure 2** outlines the experimental design investigating the role of the gut-lung axis in disease control of co-infected pigs. Specifically, FMT material was donated from 2 high-health sows and transplanted into weaned pigs for seven consecutive days prior to PRRSV/PCV2 co-infection. Control pigs received a sterile saline mock-transplant for seven consecutive days prior to co-infection. Post-infection, transplanted pigs had reduced morbidity and mortality, decreased PRRSV and PCV2 replication, improved uniformity of weight gain, an enhanced immune response as demonstrated by increased antibody production, and a decreased requirement for antimicrobial administration. This study provides evidence that microbiome modulation through FMT may be an alternative strategy in the preventative healthcare of pigs at risk for polymicrobial respiratory disease (Niederwerder, Constance et al. 2018).
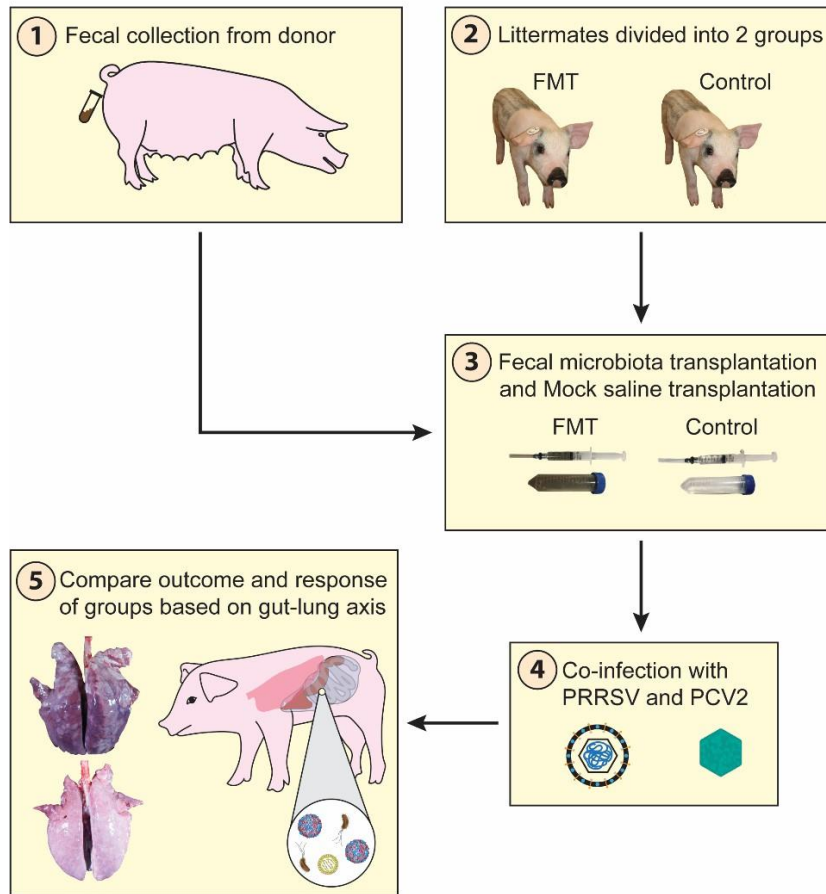
Figure 2. Experimental design to investigate fecal microbiota transplantation as a prophylactic tool to control disease associated with PRRSV/PCV2 co-infection. Figure adapted from Niederwerder, Constance et al. (2018).

Conclusions

Several gut microbiome characteristics, such as increased microbial diversity, shifts in microbial composition, and fecal microbiota transplantation, are associated with improved outcome following co-infection with PRRSV and PCV2 in nursery pigs. Improved outcome characteristics include reduced virus replication, increased antibody production, decreased lung pathology, as well as reduced morbidity and mortality. Harnessing the beneficial characteristics of high performing individuals through their gut microbiomes is an exciting, and likely underestimated, opportunity to increase the herd health of swine (Niederwerder 2018). Future research focused on understanding how microbiome modulation may be applied to improve the health and welfare of swine endemic for these and other diseases is warranted.

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Immune response after vaccination - peculiarities of the porcine immune system

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Introduction

Vaccination in pigs is widely used worldwide and is an effective means of preventing the spread of infections.
In the meantime, there are many vaccines that work very well, but often their mechanism of action is still unclear. It is known that besides antibodies and neutralizing antibodies, components of the cellular immune system play an important role.

Cells of the immune system can be differentiated by their surface antigen expression and in the last decades a multitude of these differentiation antigens (cluster of differentiation, CD) could also be defined in pigs (Lunney, 1993, Saalmüller, 1996, Saalmüller & Gerner, 2016). However, some special features have to be considered in the pig. Here, I would like to focus on three subpopulations of leukocytes: Natural Killer (NK) cells, T cells with T-cell receptors (TCR) composed of a γ and δ chain: TCR-γδ T cells and the classical CD4$^+$ T cells.

NK cells

In swine, all CD3$^-$ NK cells are CD2$^+$ (Pescovitz et al., 1988), and they also express CD16 (Mair et al., 2013). With respect to further differentiation markers, a major peculiarity occurs in the pig. In contrast to all species characterized so far, three subpopulations can be defined in the pig by the expression of the differentiation antigens CD8α and CD335 (NKp46): CD8α$^+$CD335$^-$, CD8α$^+$CD335$^+$, and NK cells with reduced CD8α expression showing high CD335 expression (Mair et al., 2012). All three subpopulations are also characterized by high perforin expression (Cossarizza et al, 2019) with CD8α^{dim}CD335^{high} NK cells showing the highest cytolytic capacity and also being the best interferon (IFN)-γ producers (Mair et al, 2013). These highly activated CD335^{high} cells appear to differentiate into the other phenotypes. An important role could be led by these NK cells through their IFN-γ production in polarizing the cellular immune response into a Th1-specific immune response, which is specifically important in the response against viruses and intracellular bacteria.

TCR-γδ T cells

Pigs, together with cattle, sheep, and chickens, belong to animal species that possess a high proportion of TCR-γδ T cells. Within porcine TCR-γδ T cells, two subpopulations can be defined by expression of the differentiation antigen CD2: CD2$^-$ and CD2$^+$ TCR-γδ T cells (Saalmüller et al. 1989, Stepanova & Sinkora 2013). Specifically, the CD2$^+$ TCR-γδ T cells are characterized by a particular affinity for lymphoid organs and could be identified as the TCR-γδ T cells that also show a distinct IFN-γ production (Saalmüller et al., 1989, Hirt et al., 1990, Saalmüller et al., 1990, Sedlak et al., 2014a, Sedlak et al., 2014b). They appear to represent more differentiated TCR-γδ T cells. However, the *in vivo* function of TCR-γδ T cells remains poorly understood.

CD4$^+$ T cells

Porcine T cells can be distinguished by a variety of differentiation antigens (Saalmüller & Gerner, 2016). In addition to CD2, which is expressed on all TCR-αβ T cells (Saalmüller et al., 1989, Pescovitz et al., 1994a), TCR-associated CD3 naturally plays an important role (Saalmüller, 1996, Pescovitz et al., 1998). Porcine CD4 is expressed by a distinct subset of T cells (Pescovitz et al, 1985, Pescovitz et al, 1994b) however also by plasmacytoid dendritic cells (pDC, Summerfield et al., 2003, Summerfield & McCullough, 2009). The differentiation markers CD5 and CD6 are found on all TCR-αβ T cells, with CD5 also showing weak expression on TCR-γδ T cells (Saalmüller et al., 1994a, Saalmüller et al., 1994b, Saalmüller et al., 1994c, Pauly et al., 1996). CD8 molecules can exist either as αα homodimers or as αβ heterodimers. CD8αβ heterodimers are exclusively expressed on thymocytes and cytolytic T cells (CTL) (Yang & Parkhouse, 1997). CD8αα homodimers are found on NK cells (Pescovitz et al., 1988), but also on activated CD4$^+$ T helper cells and memory CD4$^+$ T cells (Saalmüller et al., 1987, Saalmüller et al., 1994d, Saalmüller et al., 2002).

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Considering the phenotype of porcine T cells, four T-cell subpopulations can be defined in addition to TCR-γδ T cells based on the expression of CD4 and CD8α (Pescovitz et al., 1985, Saalmüller et al., 1987, summarized in Cossarizza et al. 2019). A small subpopulation of CD4⁻CD8α⁻ T cells, that does not carry TCR-γδ receptors, CD4⁻CD8α⁺ cytolytic T cells that are generally characterized by the expression of CD8αβ heterodimers, and within CD4⁺ T cells, CD4⁺CD8α⁻ cells with the phenotype of classical T-helper cells and CD4⁺CD8α⁺ cells that contain activated T-helper cells and memory T cells (Saalmüller et al., 1987, Summerfield et al., 1996, Saalmüller et al., 2002). By expressing CD27, CD4⁺ T cells can be further subdivided into CD4⁺CD8α⁻CD27⁺ naive T cells, CD4⁺CD8α⁺CD27⁺ central memory cells, and CD4⁺CD8α⁺CD27⁻ effector memory cells (Reutner et al., 2012, Reutner et al., 2013). These CD4⁺ memory cells are characterized by their IFN-γ and tumor necrosis factor (TNF) α production in recall responses with different antigens (Koinig et al., 2015, Talker et al., 2015, Talker et al., 2016, Schmidt et al., 2020).

As for the differentiation of CD4⁺ T cells, we must assume a CD8α⁻CD27⁺ cell, which additionally expresses CD45RC, CCR7, and CD62L. After activation, there is an expression of CD8α and MHC-II (Saalmüller et al., 1987, Saalmüller et al., 1991) and a downregulation of CD45RC. This phenotype is also characteristic for central memory cells, and upon differentiation into effector memory cells, downregulation of CD27, CCR7 and CD62L occurs.

This differentiation, which was first analyzed *in vitro* (Saalmüller et al., 2002) could also be detected *in vivo* by monitoring the development of immune cell populations in individual animals over a longer period of time. Also *in vivo*, CD4⁺CD8α⁻CD27⁺CD45RC⁺MHC-II⁻ cells become T cells with the phenotype CD4⁺CD8α⁺CD27⁻CD45RC⁻ MHC-II⁺ during their further differentiation (Talker et al., 2013). In the course of this differentiation, a polarization of the CD4⁺ T cells takes place under the influence of fate-determining cytokines. Here, so-called transcription factors come into play, which are induced by corresponding cytokine cocktails. As in other species, transcription factor T-bet positive IFN-γ producing Th1 cells, GATA-3 positive interleukin (IL)-4, IL-5 and IL-13 producing Th2 cells, RoRγT positive IL-17A producing Th17 cells (Rodriguez-Gomez et al., 2016), and Foxp3 positive regulatory T cells could be generated in pigs (Käser et al., 2008a, Käser et al., 2008b, Käser et al., 2011, Käser et al., 2012, summarized in Saalmüller & Gerner, 2016).

Conclusions

These works show that the porcine cellular immune system has some peculiarities compared to other species. Taking into account the knowledge of these peculiarities when analyzing the cellular immune response after vaccination, we are in the best position to characterize the efficiency of vaccines at the T-cell level (Koinig et al., 2015, Talker et al., 2015, Talker et al., 2016, Stadler et al., 2018, Schmidt et al. 2020).

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Moving the boundaries in understanding immunity in livestock using systems immunology

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Abstract

The complexity of the immune system is based on many functional specialized cell types that interact with a enormous array of cell surface receptors and soluble cytokines that regulate activation as well as chemokines that regulate migration. Activation and de-activation processes are equally important for a well-targeted immune response that avoids causing unnecessary tissue damage. Migration of immune cells represents another key feature of such a response and includes migration from primary lymphoid tissue to the blood, from the blood to inflamed tissues, from inflamed to issue to draining lymph nodes and from activated lymph nodes back to the blood and inflamed tissues. Understanding this complexity in space and time requires a systems immunology approach that employs "omics" technologies, data science tools and bioinformatic pipelines that provide immunologically relevant and interpretable data. Here we provide examples for such an approach and demonstrate how such technologies can be applied to veterinary species including pigs to measure and understand immune responses to vaccination and infection. This information is essential to rationally design new live attenuated vaccines, to optimize adjuvants or develop new immunotherapeutic drugs for pig medicine.

Introduction

Traditional method for evaluating vaccines and immune responses against infectious pathogens are based on specific antibody and T-cell readouts or even challenge experiments. These have a number of drawbacks that relate to three main problems. First, the animal's immune system can be see in analogy to a "black box" which records information on immunological perturbance that is difficult to access and decode. It can therefore be challenging to define immunological processes leading to protective immunity (Hagan et al., 2015;Hagan and Pulendran, 2018;Villani et al., 2018;Hagan et al., 2019). This is particularly evident for innate immune responses which represent and essential component of protective immune responses. Furthermore, innate immune responses strongly contribute to the induction of adaptive immunity. Second, we lack informative methods to measure in a comprehensive manner how environmental factors such as nutrition, stress, microbiome and hygiene as well as genetics impact immune responses. In fact, the variability between animals often observed for responses to vaccines and infectious agents is caused by such factors. Therefore, more robust immune response analyses methods are required that are less impacted by variability. This will of course also help in understanding resilience, tolerance and resistance to infectious diseases. Third, for some infectious diseases such as African Swine Fever and some bacterial infections, the immunological correlates of protection are not known and therefore current immunological readouts do not inform on the potential protective value of a vaccines.

We propose that deep immunological analyses based on the principles of systems immunology will provide solutions to the above problems. Ideally, this should include "omics" and multiplexing technologies in combination with protective, clinical data and traditional immune response data. The later can cover the whole spectrum of B- and T-cell responses such as the quantification of serum antibodies and their function, or the more difficult measurement of circulating memory B- and T-cells that usually requires in vitro re-stimulation experiment and an experienced immunology and cell culture laboratory. The large data set then needs to be analyzed using computational pipelines that inform on relevant immunological perturbations and correlates of protective immunity (Hagan et al., 2015;Hagan and Pulendran, 2018;Villani et al., 2018;Wimmers and Pulendran, 2020).

By measuring thousands of parameter, systems immunology approaches strongly enhance the statistical power of in vivo studies and therefore permit to obtain solid data despite low numbers of animals per group and heterogenous populations (Haining, 2014). Furthermore, our approach is focussing on very early time points post vaccination, informing on beneficial innate immune responses, as well as unwanted inflammation.

Considering that the type and strength of such early innate responses dictates adaptive immunity, innate immune responses may represents correlates of protection and form the basis of biomarkers to speed up the development of novel immunotherapeutics (Li et al., 2014),(Oberg et al., 2011).

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Blood transcriptional modules

A central scientific basis of our approach is that the transcriptome of peripheral blood leukocytes collected very early after vaccination or infection informs on molecular signatures of protective immune responses as well as of safe vaccines (Cortese et al., 2020). In our lab, we have established and extensively used blood transcriptional modules (BTM) to tackle the above issues. BTM are composed of highly interacting and tightly co-regulated immunologically relevant genes (Chaussabel et al., 2008;Li et al., 2014), and have been demonstrated in many studies to provide immunologically relevant data with a high predictive power. This includes information on changes in immune cell population distribution, cellular processes such as cell cycle and transcription, leukocyte-specific signalling pathways, leukocyte migration, activation of particular immune cell types such as dendritic cells, B cells and T cells, inflammation, coagulation, platelet activation, antiviral responses, antigen presentation, immunoglobulin production and metabolic processes relevant for immune responses (Li et al., 2014;Li et al., 2017;Hagan et al., 2019). We have successfully adapted the BTM system originally established for human leukocytes to veterinary species including sheep, goats and pigs (Braun et al., 2018;Matthijs et al., 2019;Eloiflin et al., 2021). Similar to human studies, the work in pigs, goats and sheep demonstrated its efficiency in detecting and explaining immunological processes occurring in blood and tissues. For instance, as early of one day following vaccination, it is possible to identify BTM correlating to adaptive immune responses measure several weeks or months later. Therefore, we can predict vaccine responses. We also demonstrate that such as methodology enables a detailed characterization of immune responses induced by different formulations (Matthijs et al., 2019). Our data has also highlighted that conserved correlations patterns of BTM with adaptive responses can be found across multiple species (Braun et al., 2018;Matthijs et al., 2019;Cortese et al., 2020).

Results obtained with inactivated vaccines

Published and unpublished data demonstrate that the BTM systems immunology pipeline can help to dissect the impact of vaccine components and formulations on the immune system and thereby help to identify improved delivery systems and immunostimulants. We have demonstrated this for foot-and-mouth diseases vaccines and bacterin mycoplasma hyopneumoniae vaccines (Braun et al., 2018;Matthijs et al., 2019;Cortese et al., 2020). The data demonstrates that it is possible to identify innate immune patterns in the blood reflecting activation in tissues. This also informs on immune cell migration patterns such as from the bone marrow to the blood, from the injection/inflammation site, and from the draining lymph node. Considering that such migration patterns correlate with adaptive immune responses including both T- and B-cell responses, we postulate their involvement in the induction of immune responses. Another important observation was that our studies in pigs and sheep clearly indicated that very early innate immune responses dictate late adaptive responses in a comparable manner across multiple species. This represents a basis for rational improvement of vaccine adjuvants.

The general profile of correlating modules observed is schematically represented in Figure 1. Typically, innate BTM belonging to the family of IFN type I BTM, inflammatory BTM and myeloid cell BTM are strongly induced by potent vaccines. Depending on the adjuvant, the profile can be more inflammatory or antiviral. Importantly, a counter regulation below baseline expression observed around one week post vaccination also represents a correlate of good vaccines demonstrating the importance of regulating innate immune responses (Figure 1A). For BTM indicative of adaptive immune responses an opposite profile typically correlates with adaptive antibody and T-cell responses. This means an early downregulation followed by an upregulation of T-cell and cell cycle BTM. As expected, plasma cell BTM represent a good correlate for circulating plasma cells which themselves correlate with serum antibodies detectable a few weeks later.

Days post vaccination
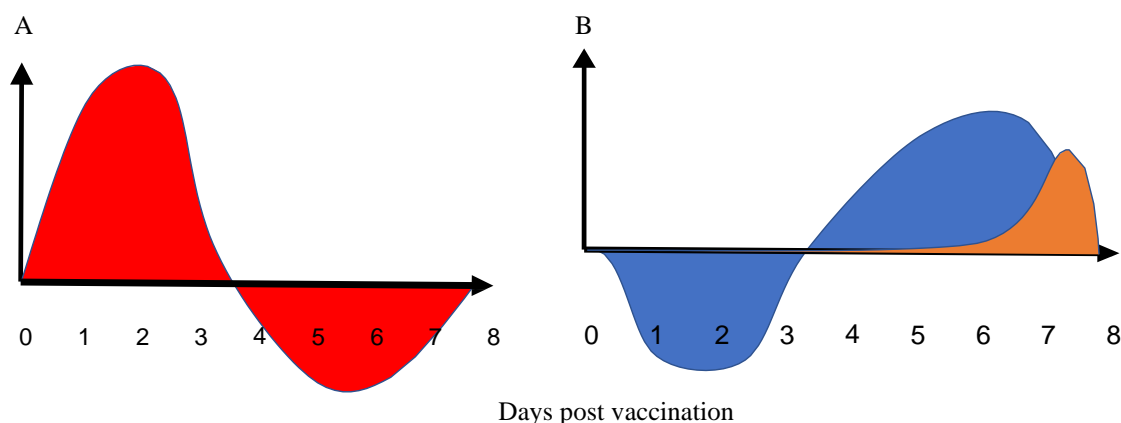
Figure 1. Schematic representation of innate correlates of potent inactivated vaccines identified using the BTM transcriptomic pipeline. A. Correlation profiles for innate BTM containing IFN type I, inflammatory, dendritic cells and myeloid cell migration modules. B. Correlation profiles for adaptive BTM containing T-cell (blue), cell cycle (blue) and plasma cell (orange) BTM. X-axis: days post vaccination; y-axis: strength of response above and below baseline.

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Results obtained with live vaccines and infections

The correlation patterns of BTM to adaptive immune responses following vaccination with inactivated vaccines differs from that following infection or live attenuated vaccine (LAV) application as demonstrated for several human vaccines (Li et al., 2014). As BTM expression is likely to strongly differ between different viruses, and we do not yet have sufficient data, to propose a general "thumb" rule as given in Figure 1 for inactivated vaccines. Therefore, Figure 2 summarizes only our findings for porcine reproductive and respiratory syndrome virus (PRRSV) infection focusing on BTM correlating to T-cell responses (Bocard et al., 2021).

	day 3			day 7			
	innate BTM	adaptive BTM		innate BTM		adaptive BTM	
Th/Treg	DC	B cells*	T/NK cells	IFN-I	inflammation myeloid cells	T/NK cells	cell cycle
Tc	DC	B cells			inflammation	T/NK cells	cell cycle

* only for T cell responses at day 42 or later

Figure 2. Schematic representation of correlates of T-cell responses following infection by PRRSV. Blue colors indicate a downregulation of the BTM correlating with T-cell responses (either cytotoxic T cells (Tc) or T-helper and T-regulatory cells (Treg). Red colors indicate an upregulation of the BTM correlating with T-cell subsets, as indicated. Color intensity indicate the degree of correlation (data from (Bocard et al., 2021)).

An early downregulation of dendritic cells and B-cell BTM were found to generally correlate with all classical T-cell responses. An upregulation of T and natural killer cell (NK) cell modules at day 3 was a correlate of T-helper (Th) and T-regulatory (Treg) cells. In contrast to the inactivated vaccines, innate BTM upregulated at day 7 positively correlated with T cell responses. In common with inactivated vaccines was the positive correlation of T-cell and cell cycle BTM with T-cell responses.

Understanding the impact of hygiene and the microbiome

Application of a systems immunology approach to characterize innate immune responses following infection with African swine fever virus (ASFV), has demonstrated that the balance of pro- and anti-inflammatory responses early during infection plays an important role for the outcome of infection. We found that specific pathogen-free (SPF) pigs, which differed strongly in their microbiome composition when compared to farm pigs, also differed in their onset, intensity and counter regulation of inflammatory responses when compared to farm pigs. This appears to be caused by a different baseline of immune activation between the groups of pigs. In fact, farm pigs had a higher inflammatory and antiviral baseline, which delayed viremia development but was associated with a more intense systemic "cytokine storm" after infection with a highly virulent ASFV strain, when compared to SPF pigs. With a partially attenuated ASFV strain, the higher anti-inflammatory status of SPF was associated with a more efficient recovery from infection when compared to farm pigs. Thus, the hygiene-dependent immune baseline represents a double-edged sword during African swine fever.

More generally, the data demonstrates that the BTM-based systems immunology pipeline is suitable to identify pathways responsible for the heterogeneity in immune responses such as the impact of hygiene and microbiome.

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Intrauterine immunization as a safe and effective route of immunization in gilts and sows

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Mucosal Immunization in pigs

Vaccines against infectious diseases are critical for protecting pigs, especially in industrial barns where animal numbers tend to be high. Traditionally, vaccines have been administered using a needle which requires snaring the animal or using a needle-gun device. Delivery of vaccines in large animals such as pigs can be difficult and a potential safety hazard, as observed by the large portion of swine veterinarians who report needle-stick injuries, with 40% of those injuries occurring when delivering vaccines (A. L. Hafer et al., 1996). And with the move towards group housing, the free mixing and movement of animals will further increase difficulty in the ability to safely administeringneedle-based vaccines and tracking which animals have been immunized.

An alternative to using needles is the delivery of vaccines through mucosal routes including by mouth (i.e. in water or feed), into the eye, or intranasally (Atul Srivastava et al., 2015; Volker et al., 2006). Because most mucosal- delivered vaccines do not require needles, their use will reduce the number of vaccine-related injuries acquired by veterinarians and swine staff (A.L. Hafer et al., 1996). A successful mucosal-administered vaccine administered at one location will induce an immune response at other mucosal sites as well as in the blood (Gerdts et al., 2006; A. Srivastava et al., 2015). As the vast majority of pathogens enter the body through these mucosal sites, a strong mucosalimmune response has the potential to eliminate pathogens prior to them crossing the epithelial barrier, and can, in thecase of the uterus, protect the developing fetus from pathogens. However, to design a successful mucosal vaccine, several challenges are encountered such as avoiding vaccine elimination by the flow of mucosal fluids across mucosalsurfaces, recruitment and targeting of antigen presenting cells (APCs) and establishing a suitable dose and volume forthe vaccine to traverse the epithelial barrier (Woodrow et al., 2012a, 2012b) without inducing a tolerogenic immune response (such as is required for antigens in microflora, food, and environmental particles (Czerkinsky et al., 1999)).

While oral, ocular and intranasal vaccines may be practical for delivering vaccines to piglets, there is a greater degree of difficulty in delivering these vaccines to full-grown animals such as gilts and sows because their administration may require restraint of the animals. We are investigating administering vaccines into the pig uterus as an alternative mucosal route which has several advantages over other mucosal routes. When gilts or sows are in estrus and in proximity to a boar or its pheromone androstenone, they undergo a lordosis response where they become rigid in preparation for mounting (Dorries et al., 1997). Breeding of sows by artificial insemination (AI) is already an established practice in commercial pig operations and it takes place multiple times per year, this route offers a safe and easy means of incorporating a new vaccination regimen into current husbandry practises (Knox, 2016; McNamara et al., 2013) making the uterus readily accessible at every breeding cycle. With all these considerations, intrauterine immunization should be explored as an alternative route of immunization however, they require careful selection of adjuvants and delivery methods to develop safe, effective and economically viable vaccines.

The uterous as an immune inductive site

While the female reproductive tract in sexually mature animals is a dynamic system that undergoes several modifications in response to the hormonal status of the animals throughout the estrous cycle, we are intent on investigating the effect of intrauterine immunization only at standing estrus when the cervix is permissive. A permissive cervix may allow for the introduction of pathogens in the semen and/or induction of immunity in response to a vaccine. Characterization of cells or responses in the reproductive tract outside of the estrous cycle, while interesting, will not advance our goal of establishing a husbandry practise coupled with vaccination.

The uterus has an active immune response to semen, seminal plasma, and bacteria or viruses (Bischof, Lee, et al., 1994) and this response is critical to clear excess sperm (Katila, 2012). Breeding in swine elicits an inflammatory immune response resulting in release of cytokines and chemokines into the lumen (Katila, 2012; Rozeboom et al., 1999). Another study showed that the semen extender Androhep and seminal plasma alone induced IL-10, TGF-β,

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IL-8, and TNF-α, however when combined with spermatozoa, the level of gene expression of these genes was reduced suggesting that spermatozoa may contribute to a degree of suppression (Taylor et al., 2009). There is significant leukocyte recruitment to the uterine lumen (including APCs) in response to seminal plasma and semen extenders but the majority of the cells recruited are polymorphonuclear cells (Rozeboom et al., 1998), which are not the target cell population for an intrauterine vaccine (Hamonic et al., 2020). Adjuvants that promote APC recruitment to the lumen or direct stimulation of the uterine epithelial cells lining the uterus may be key to an effective intrauterine vaccine administered with extended semen.

The outer layer of the porcine uterus is composed of a columnar epithelium across the entirety of the uterus, however, unlike the vagina and cervix, there are also glandular epithelial cells which form tubular glands that spiral into the tissue (Hussein et al., 1983). A highly vascularized layer of connective tissue can be found below the epithelial layer, which together makes up the endometrium which changes in thickness and epithelial cell height during the estrus cycle (Edstrom, 2009; Kaeoket, Persson, et al., 2002; Lorenzen et al., 2015). Epithelial cells are the outermost layer of cells of all mucosal surfaces and the skin and they form tight junctions between adjacent cells that form a barrier preventing the entry of pathogens into the body (Nusrat et al., 2000). These cells impact the immune system through the expression of several pattern recognition receptors (PRRs) and they induce cytokine and chemokine secretion in response to pathogen-associated molecular patterns (PAMPs) stimulation, resulting in the initiation of immune responses of pathogens at the epithelial barriers (Wira, Grant-Tschudy, et al., 2005). The role of uterine epithelial cells as sentinels for the uterine immune response has been much more widely studied in other species including humans (Wira, Grant-Tschudy, et al., 2005) where UECs responded to immunostimulation with cytokine and chemokine secretion and subsequent immune cell recruitment (Schaefer et al., 2004; Soboll et al., 2006). Porcine UECs stimulated with polyI:C and CpG showed induction of IL-6 and IL-10, respectively which shows conservation of cytokine expression profiles with studies in mice and humans uterine epithelial cells whereof the two only polyI:C induced pro-inflammatory cytokines (Fahey et al., 2008; Hamonic et al., 2018; Soboll et al., 2006). In addition to cytokine and chemokine secretion in response to PRR signalling, porcine mucosal surface epithelial cells secrete β- defensins to directly eliminate microbes (Nijnik & Hancock, 2009). In humans and mice, it has been determined that select epithelial cells have the capacity to express MHC class II and to present antigen directly to T cells (Mulder et al., 2011; Wallace et al., 2001; Wira, Rossoll, et al., 2005). However, thus far no study in pigs have detected MHC class II expression by epithelial cells (Mair et al., 2014) which highlights species-specific differences between the role of epithelial cells in immune activation. Thus, porcine uterine epithelial cells maintain the capacity to act as sentinels for the detection of incoming pathogens due to the conservation of PRR expression and localization patterns with other species uterine epithelial cells.

The uterine epithelial barrier is devoid of specialized epithelial cells such as M cells, which are efficient at sampling and delivering antigens to underlying immune cells in the small intestine (Kim & Jang, 2014; Woodrow et al., 2012a). The endometrium is reported to have semi-structures called lymphoid aggregates present throughout the endometrium that are typically located directly basolateral to the glandular epithelium (Lorenzen et al., 2015). The uterine tissue contains a multitude of both luminal (Kaser et al., 2017) and subepithelial lymphocytes (Bischof, Brandon, et al., 1994). Immune cells in the endometrium are primarily lymphocytes, and although numbers will vary throughout the estrus cycle, typically CD8+ T cells are present in high numbers below the surface epithelium, with more CD4+ T cells deeper within the connective tissue (Kaeoket, Dalin, et al., 2002). Plasma cells are dispersed throughout the endometrium with a predominance for IgG-secreting plasma cells (Hussein et al., 1983). Lastly, the distribution of uterine antigen presenting cells (APCs) such as macrophages and DCs are present throughout the endometrium during estrus, however at other stages of the estrous cycle, they are found deeper in the lamina propria and rarely reside directly below the surface epithelium (Kaeoket, Dalin, et al., 2002; Kaeoket, Persson, et al., 2002; Wira, Fahey, et al., 2005). As the uterus has no mucosa-associated lymphoid tissue (MALT), trafficking of the mature DCs and presentation of antigen to T cells occurs in the draining lymph node present in the broad ligament (Woodrow et al., 2012b). Likewise, vaccine formulation and delivery can be directed towards normal epithelial cells or at immune cells recruited to the uterine lumen or tissue.

Considerations for using semen to deliver intrauterine vaccines

The goal of all vaccines is to generate high enough population-level immunity that results in decreasing the circulating pathogen levels that protect those most at risk through herd immunity (Fine, 1993). Subunit vaccines are considered safer as there is no whole pathogen delivered (and therefore no danger of reversion to virulence), however, they are typically less immunogenic and require adjuvants to increase their immunogenicity (Bastola et al., 2017). Adjuvants facilitate uptake of the antigen across the epithelial barrier, recruitment of APCs, activation of APCs, directing of the immune response (Th1, Th2, Th17-type T cell or CTL), creating a depot and protecting the antigen from degradation (Awate et al., 2013; Pashine et al., 2005; Srivastava et al., 2015). One or several of these mechanisms of action may be required to generate a successful mucosal vaccine response and, therefore, the inclusion of multiple adjuvants may be necessary for an effective vaccine formulation (Garg et al., 2017). However, because this intrauterine vaccine is

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being administered in the presence of live semen, the adjuvants and antigen combination must not negatively affect sperm function or motility. When we initially started investigating combining vaccines with semen, we combined several commercial pig vaccines at very low doses with semen and they were all spermicidal. We therefore took steps to find adjuvants that were not spermicidal. We performed computer assisted sperm analysis and microscopy with peanut agglutinin which binds to acrosome reactive sperm. By testing a dose titration of several adjuvants in commercial extended semen, and we found that the host defense peptide, poly IL:C and polyphosphazene (TriAdj) plus inactivated PPV and recombinant proteins did not significantly impact sperm function (Hamonic et al., 2020). Results from subsequent trials have shown that the i.u. vaccine did not appear to affect sperm function or fertility as pigs immunized at breeding with semen plus recombinant PPV antigen or FliC antigen and/or inactivated PPV plus TriAdj had healthy fetal development and healthy piglets with no obvious difference in average daily gain up to weaning. Several more trials will need to be performed with much high numbers of dams before we can stake this claim with certainty. While these results were only based on a few trials and only a few dozen animals, i.u. vaccines formulated with TriAdj did not appear to negatively affect sperm function, embryo viability or at least short-term piglet growth kinetics (Choudhary et al., 2021; Hamonic et al., 2020).

When we considered administering an intrauterine vaccine into livestock such as pigs, there is the concern that there is the possibility of generating an immune response to sperm that results in infertility or reduced fertility in future pregnancies, as has been observed in in humans, mice and rabbits following immunization with sperm specific proteins (Clark & Schust, 2013). The effect of several dozen i.u. immunizations and breeding cycles should be something that is carefully monitored because any reduction in fertility would not be viewed favourably by the industry.

Intrauterine immunizations in animal

Administration of a single administration of a vaccine into the uterus of rats has been shown to trigger antigen-specific systemic and local antibody-mediated immunity (Wira & Sandoe, 1989). Ovariectomized rats twice immunized by placing sheep erythrocytes directly in the uterine lumen showed pronounced IgA and IgG antibody responses in uterine and vaginal secretions and measured 2 weeks after the booster. Vaginal antibody levels (but not uterine antibody levels) were lowered by estradiol treatment indicating that this response was hormonally dependent. IgG but not IgA antibodies were also found in saliva of the twice immunized animals. Estradiol had no effect on salivary IgG levels in contrast to those of the genital tract. To study the effect i.u immunization in another animal model, we performed surgery to introduce 400 ul volume into each rabbit uterine horn consisting of ovalbumin (OVA), truncated glycoprotein D (tGD) from bovine herpesvirus, and a fusion protein of porcine parvovirus VP2 and bacterial thioredoxin (rVP2-TrX) each formulated with a TriAdj (Pasternak et al., 2017). Control rabbits received one of the subunit vaccines via the i.m. route. Vaccination through either route-induced antigen-specific humoral responses systemically and within the local (uterus) and distal mucosa (lungs and vagina). The observed mucosal response was not compartmentalized to, or within, the upper genital tract and the degree of response appeared to be at least in part antigen dependent. In a follow up study, two vaccines formulated with tgD or OVA, each formulated with TriAdj were delivered at varying doses to the uterine lumen of rabbits and the resulting immune response was evaluated 32 days later (Pasternak et al., 2018). Intrauterine vaccination produced a dose-dependent induction of both antigen-specific IgG and IgA in serum and the uterine and broncheoalveolar lavage of the high and medium-dose vaccines triggered a significant increase in both anti-OVA and anti-tgD IgG, but not IgA antibodies. The restimulation of splenocytes from the high-dose vaccine group with ovalbumin (OVA) only resulted in a small but significant increase in gene expression of the Th1 cytokines (IL2/IFNgamma) in the absence of an observable increase in proliferation. We must point out that the hormone levels of the rabbits and/or any signs indicating they were in estrus were not recorded so it is likely that not all animals were in the same stage of the estrous cycle. Regardless, the high dose of antigen triggered immunity in all animals. Collectively, the results confirm the capacity of the uterine immune system to generate a primary response following stimulation that can trigger both systemic and mucosal immunity when administered into the uterine lumen.

We initially studied whether the pig uterine immune system could respond to booster vaccines (Hamonic et al., 2020). Prior to each breeding, pigs received an intramuscular commercial vaccine containing inactivated porcine parvovirus. After gestation, farrowing and weaning, we administered our intrauterine vaccine at the next breeding. We formulated killed PPV with an adjuvant combination of TriAdj and administered it into the semen bag immediately prior to breeding these multiparous sows and the control sows received a commercial Parvovirus vaccine via the i.m. route as indicated by the manufacturer. The control and i.u.-vaccinated sows showed comparable anti-PPV IgG, IgG1 and IgG2 antibodies in serum. We followed up by administering a single i.u. vaccine into the uterus during breeding of gilts that had not received any i.m. vaccines against PPV (Hamonic et al., 2020). Results from a single i.u. vaccination with inactivated PPV plus TriAdj failed to trigger robust systemic or mucosal antibody-mediated immunity. We speculated that the semen may have negatively impacted the immune response because rabbit uterus clearly induced robust immunity after a single immunization. Two other reasons may be that multiple immunizations may be needed to promote robust mucosal immunity in the pig and/or that the triple adjuvant failed to overcome the mucosal barriers and initiate recruitment of APCs to the mucosal surface in the pig.

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To address whether multiple immunizations induced the mucosal immune response, we immunized gilts 3x into the uterus at estrous during breeding with recombinant PEDV Spike protein (PEDVS) formulated with TriAdj (Choudhary et al., 2021). The gilts were administered the vaccines with semen that was repeatedly freeze-thawed to kill them without rupturing the sperm. Because the gilts were not pregnant, they entered estrous approximately 3 weeks later as expected. This mock-breeding was repeated and then they receive the i.u. with live semen at their 3rd breeding cycle. This approach was used to shorten the length of time between immunizations as an artificial means and obviously the purpose of i.u. immunization would be to take place at each pregnancy as needed. The gilts continued to return to estrous, and they had comparable numbers of live births relative to control gilts confirming that the intrauterine vaccine administered with semen did not affect sperm function, fertility, not did it contribute to an obvious anti-sperm immune response. The thrice i.u.-vaccinated pigs had elevated anti-PEDVS antibodies in serum, in the uterus, and in colostrum as well as significantly elevated anti-PEDV neutralizing antibody titres. Significant anti-PEDVS neutralizing antibodies were also present in the colostrum which was passively transferred to the suckling piglets. The piglets born from the i.u. vaccinated gilts survived approximately 1 day longer than the piglets born from control gilts, suggesting partial protection against infectious PEDV. We suspect that the neutralizing antibodies in the colostrum were not sufficiently high to ultimately protect the piglets from the disease.

Studies are underway to establish whether replacement of the TriAdj with more robust mucosal (but non-spermicidal) adjuvants could augment the immune response to such a degree that it would protect piglets against infectious PEDV or other neonatal diseases or protect the dam against reproductive disease such as PRRSV or PPV. Studies are also underway to determine how the uterus can act as an immune induction site. Specifically, we will clarify whether the vaccine triggers immune cell recruitment into the lumen that is critical for induction of immunity or whether the antigen traverses the uterine wall to trigger immunity. How it traverses the gut wall (paracellular or transcytosis, uptake by dendritic cells extending dendrites into the lumen, etc.) and whether the antigen is presented to draining lymph nodes or lymphoid aggregates will also be investigated. Once it is clear how the uterus acts as an immune induction site, we can formulate the vaccines to exploit this mechanism of action.

In conclusions, we are encouraged that the i.u. immunization in pigs does not appear to affect sperm function, fertility or piglet growth kinetics and that it triggers systemic and mucosal immunity. Studies need to be expanded to include a broad range of adjuvants that can be formulated, perhaps as nanoparticles, that do not kill sperm. Once accomplished, extensive studies must be performed to show that robust cellular and antibody-mediated immunity can protect the sow from infectious disease and/or the suckling piglets can be protected with neutralizing antibody titres in colostrum. The industry will surely benefit from a mucosal vaccine that can be administered during lordosis when the dam is immobilized and by coupling immunization with the already accepted husbandry practise of breeding.

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Reducing antimicrobial use in pig production through improvement of management and biosecurity

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Introduction

Antibiotics knew their first applications in humans, revolutionizing the practice of medicine and gaining early on the nick name "miracle drugs". The discovery of antimicrobials also had their impact on Veterinary medicine, adding to a better health and welfare in animals (Economou & Gousia, 2015). The discovery and early use of antibiotics in animal production coincided with the post-war revolution from small scale extensive livestock production systems to a more intensive industrialized livestock production, where animals were housed indoor in large flocks and herds (Gustafson & Bowen, 1997; Steinfeld, 2004). This evolution not only resulted in farmers using more metaphylactic and prophylactic medication, it also paved the way for the commercial application of antibiotics as growth promotor. Although early studies on the impact on production of antibiotics given in the feed as growth promoters indicate productivity gains ranging from 1% to double digits, depending on factors such as nutrition, breeding, housing, sanitation, as well as husbandry and management practices (Whalstrom et al., 1956; Hanson et al;, 1955; Barber et al., 1958), more recent studies have concluded that the current productivity benefits from the use of antimicrobial growth promotors in the feed have declined as the result of the adoption of modern production and management practices (Laxminarayan et al., 2015). Hence, AGPs have benefited poor management systems but they should have no place in modern animal production as they promote antimicrobial resistance (AMR). Indeed, the use of AGPs gradually phased out in Europa and, in 2003, the EU decided to ban all AGPs by 2006 (EC, 2006; Wielinga et al., 2014). In contrast to some expectations, this did not result in a substantial decline in food animal production in Europe. Although the first ban in Sweden did lead to some initial animal health problems, this was successfully addressed by improved management and disease prevention (Swedish Ministry of Agriculture, 1997). Also in other countries the increase of therapeutic use of antimicrobials after the ban turned out to be only temporary and was even non-existent in other countries such as Norway (Bos et al., 2013; Grave et al., 2004; 2006).

Insight in antimicrobial use in pig production

Since the ban on AGPs in Europe focus has shifted to the therapeutic, metaphylactic and prophylactic use of antimicrobials in animals. When studying this use some general findings are described such as a huge difference in AMU over the course of the production cycle, with the majority of the use in young pigs (Figure 1). Other typical findings include a large variation in AMU between farms within the same country and the frequent application of prophylactic medication, often with important contributions of critically important antimicrobials such as 3° generation cephalosporin's and fluoroquinolones. In addition, farm size, veterinarian, poor biosecurity and farm health management, have all been described as drivers for AMU. Furthermore, it was observed that AMU was significantly associated across age categories, indicating that farms with a high use in piglets also used more antimicrobials in their finishers. This may, among other things, be explained by farmers' habits and behaviour (Visschers et al., 2016). But above all, the study showed surprisingly large differences in AMU between the countries (Sarrazin et al., 2019). These differences between countries, but also between herds, might be related to the differences in disease prevalence or differences in the level of biosecurity. However, they may also reflect variations in rules and regulations in the countries and/or a certain attitude towards AMU of farmers and veterinarians not necessarily linked to the true animal health situation.
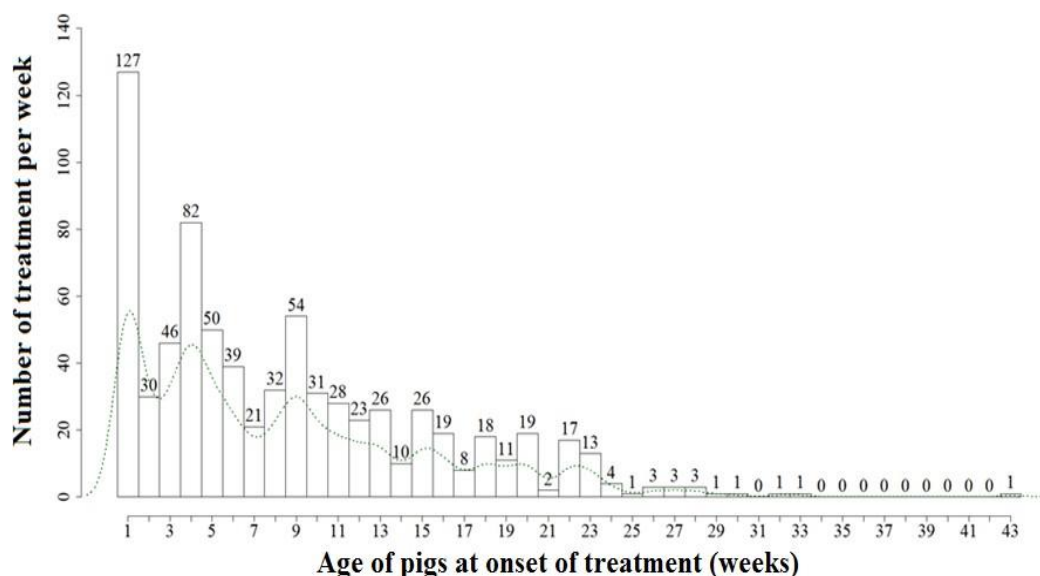
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Figure 1: Histogram showing the number of antimicrobial group treatments per week applied to a batch of pigs frombirth to slaughter based on 750 treatments (30 instances of treatments missing). The green dotted line represents the weekly average treatment incidence (adapted from Sarrazin et al., 2019).

Reducing antimicrobial use in pig production

Better health management and biosecurity

While the principal role of AMU in food animals should be therapeutic, in reality use has been substantially driven by the objective of improving farm productivity and income. High AMU has often been used as a tool to compensatefor poor health management on the farm. However, these are not acceptable practices. Therefore, husbandry systems, production systems and both management and biosecurity standards should be designed in such a way that the need for antimicrobials becomes exceptional or even redundant.

In a study by Postma et al. (2015), European veterinarians active in pig production were asked what they consider to be the most valid alternatives for AMU in pig production, taking into account expected effectiveness, feasibility and return on investment of the measures. Results indicated that practitioners believe the most promising alternatives to AMU are, in order of priority: improved biosecurity, increased and improved vaccination, use of zinc (against *E. coli* infections in weaned pigs), improved feed quality and improved diagnostics. Besides the use of zinc, which is banned for medicinal use in Europe since the end of 2021 (EC 2017), all the described alternatives are within reach for all pig producers.

In the meantime, several studies have found that improved biosecurity may result in reduced AMU, without jeopardizing production results. In a study in farrow-to-finish pig herds in Belgium, it was found that herds with higher internal biosecurity scores, determined by means of the Biocheck.UGent scoring system, had lower antimicrobial treatment incidences, suggesting that improved biosecurity might help in reducing the amount of antimicrobials used (Laanen et al., 2013). In a French study in farrow-to-finish herds, biosecurity measures such as disinfection of the loading area, gilt quarantine and adaptation, farm structure/working lines and all-in/all-out practices were found to be significantly associated with lower AMU (Lannou et al., 2012). In a multi-country study comprising four European countries, it was shown that a higher weaning age (>24 days), a batch management system of five weeks or more and the external biosecurity level were significantly associated with a lower antimicrobial treatment incidence (Postma et al., 2016). This finding was confirmed in a study of the characteristics of top pig farmers. In this study, the level of internal biosecurity was positively associated with a better control of infectious diseases and a lower need for antimicrobials (Collineau et al., 2017). In Denmark, farmers and their veterinarians implemented measures that managed to reduce their annual antimicrobial consumption by 10% or more following the introduction of the "Yellow Card system". It was reported, among other parameters, that cleaning procedures, adequate action regarding diseased animals (e.g. an earlier decision to euthanize) and all-in/all-out systems were mentioned by farmers and veterinarians as means to reduce AMU (Dupont et al., 2017). Another study concluded that improved biosecurity, especially the presence of a hygiene lock, and pest control by a professional company, were related to lower probabilities of farms being infected with extended spectrum beta-lactamase producing *E. coli* (Dohmen et al., 2017). An intervention study in Belgium found that improving pig herd management and biosecurity status, in combination with antimicrobial

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stewardship, helped reduce AMU in pigs from birth till slaughter by 52%, and in sows by 32% (Postma et al., 2017). In the latter study, the management and biosecurity interventions were generally relatively simple for farmers to implement. They included changing the working habits and routines of the farmer (e.g. changing of needles, hand and personal hygiene, and analysis of water quality). Interventions incurring higher costs and/or requiring more pronounced changes, such as introducing a new hygiene lock to change clothes/boots and wash hands, were implemented less frequently. A key recommendation was having a good and early registration of disease signs allowing to take proper and timely control measures (e.g. biosecurity, vaccination and climate change), and to create awareness of the importance of the principle that "prevention is better than cure". An economic evaluation based on the results of this study has shown that, including labour costs of all persons involved (including the coach, veterinarian and farmer), the participating herds achieved an average financial gain or overall benefit of €2.67 per finisher pig per year from partaking in this "team effort" approach (Rojo-Gimeno and Postma et al., 2016). In a comparable study performed in four European Union countries, an economic evaluation of suggested interventions in, among others, biosecurity resulted in a median change in net farm profits among Belgian and French farms estimated at €4.46 and €1.23 per sow per year, respectively (Collineau et al., 2017).

Towards zero antimicrobial use

Raised Without Antibiotics (RWA) is a certification mark that is known in countries such as Denmark and the United States. However, specific inclusion criteria for RWA production and the implementation of RWA in a large number of herds with varying management and housing conditions has only been limitedly investigated. In a recent Belgian study (not yet published) twenty-eight Belgian pig herds were enrolled and their AMU as followed for a period of 35 months. The goal of the study was to evaluate to what extend pig farms could be coached towards antimicrobial free pig production and to what extend they could also maintain this status over time. In this study RWA was defined as no antibiotics from birth until slaughter. Pigs requiring an individual treatment received a special ear-tag and were excluded from the RWA program. The results of the study showed that 13 out of the 28 herds were successfully raising pigs without antibiotics after a coaching period of one year. One year later still 12 out of the 13 were maintaining this status. Remarkably RWA herds applied less vaccinations, were smaller (median 200 sows, range 85 – 300 compared to non-RWA herds median 350 sows, range 180 - 1250), applied more frequently a 3- and 5-week batch farrowing system, compared to the 4-week system which was used significantly more in non-RWA herds. The weaning age was slightly higher (not significantly) on RWA farms (mean 24.9 days) compared to non-RWA farms (mean 23.9 days). This study showed it was possible for farmers to achieve and maintain the RWA status through herd-specific coaching related to prudent AMU and biosecurity.

Conclusions

Based upon the described evolution in AMU in pig production and based upon the results already today obtained by the leading producers and countries, it is clear that the use of antimicrobials will further diminish and ultimately will become an exceptional act in future pig farming. Obviously, for the majority of the farms both within and beyond Europe this will require further efforts and focus on better husbandry, biosecurity and management. Ultimately this reduction will also result in the levelling off, and eventually even reversal, of resistance selection, leading to further benefits for animal health as well as human health, global food safety and food security.

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How to apply science to the field:
Is PRRS our worst enemy or best mentor?

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Introduction

We, as swine veterinarians, are basically/traditionally trained as "scientists". However, once you go out to the field, you are often expected/demanded to be a "problem solver". Solving problem (especially related to health), often requires you not only scientific knowledge but also practical capability, economical consensus, and communication skills in person.

In my opinion, here are the core questions you should ask yourself every time you visit your client farm. Is your advice:
1) Science based?
2) Practically feasible (simple and organized)?
3) Effective (cost vs. benefit)?
4) Committed to be continued (execution)?
5) Measurable?

As a swine veterinarian (as well as a "problem solver"), I believe I am not the only one who always feels the dilemma between "science" and "practice" in the field. How should you apply your scientific knowledge/experiences to solve problems in the field by achieving the core questions described above?

To me, PRRS is what gave us the answer to solve such dilemma in the field. Is PRRS your worst enemy or greatest mentor? I would say; "Yes, both".

As I recognize my role of my talk as a keynote of this session; Clinical Cases of this Congress, the objective of this paper is to provide PRRS as an example to open our discussion of how we can apply/utilize science to the field.

Contents: What lessons have we leanrt from PRRS?

1. Herd imunity
   - Sow herd immune stabilization
   - Gilt immune management
   - Definition of sow herd immune stabilization
2. Production system and pig flow
   - Multi-site production system
   - All-in All-out
   - Capability of partial depopulation
   - Capability of herd closure
3. Diagnosis/testing/monitoring
   - Serology (ELISA) and antigen detection (PCR)
   - Ante-mortem investigation (necropsy and pashological examination)
   - How to interpret the results
   - Inovative tools such as Oral fluid sampling (OF), Processing fluid sampling(PF)
   - Environmental sampling
4. Biosecurity
   - Understanding the transmission routes/risks of the pathogen
   - External biosecurity (biosecurity between farms)
   - Internal biosecurity (biosecurity within the farm
   - Feed risk: *"Biosecurity is the global issue"*

5. Execution/communication
   - Audit
   - Training/education
   - Motivatiation
6. Elimination vs. Control
7. Area regional aproach
8. Global collaboration

 Much more details will be discussed in the presentation at the congress.

Conclusions

 As a summary of this paper:
1) Swine veterinarians are required to have not only scientific knowledge but also practical capability, economical consensus, and communication skills in person.
2) PRRS is a good example for us to learn those lessons.
3) Feed risk of pathogen transmission. Recent research/field investigation reveals that *"Biosecurity is the global issue"*.
4) Swine veterinary medicine/practice can be very flexible depending on country, regions, and its culture. Understanding the background conditions why it does work (or does not work) is the key for the success of the global collaboration.

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Precision Feeding during Gestation for High Producing Sows: a break through towards sustainability and productive efficiency

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Introduction

Advances in pig production have brought many benefits for producers, such as higher number of piglets/ sows per year, higher farrowing rates per year, decreased age at slaughter, increased feed efficiency and higher lean gain (Ball et al., 2008). Nevertheless, negative characteristics, such as within-litter birth weight variation have developed, resulting in economic losses and lower profits to the producer (Wolf et al., 2008). The establishment of a proper nutrition program for modern sows, should consider the genetic material of the farm, nutritional needs, factors that affect these needs, and should have the understanding of the various aspects of the metabolic interaction between genotype, nutrition and reproduction in sows. This understanding is essential so that we can achieve productivity and longevity at the same time. The gestating sow's energy state can directly influence its performance during lactation. Excessive energy can cause obesity at birth, leading to reduced voluntary intake, resulting in high physical losses during lactation (Sinclair et al., 1998; and Kim et al., 2009). The reduction in voluntary intake is to be an even bigger problem when it comes to the gilt and second parity sow in relation to adult sows. Young sows generally have a lower voluntary intake capacity, at around 20% lower (Young et al. 2004) compared to adult sows. A severe energy deficiency can result in sows being thin at farrowing, which can lead to problems during lactation with reduced capacity to produce milk and reduction in litter weight at weaning. To control the energy consumption of the gestating sows, restricted and/or controlled feeding strategies are put into practice more efficiently. The maternal gain should be recognized as the net gain in weight of the sow during the gestation period, disregarding the weight gain attributed to the uterus, placenta, placental fluids, foetuses and mammary gland. The contribution of maternal gain to the energy requirement of the sow is variable and is related to the growth phase, which is higher in primiparous sows. The total energy demand of the pregnant sow would also depend on the body condition of the animal at the time of insemination. Therefore, sows with lower body fat reserves require more energy to reach the body condition recommended for the time of farrowing.

Current Feeding Strategies for Gestating Sows and Their Impacts on Nutritional Requirements

Due to the fact that sows are fed restrictively during gestation, they may become deficient in amino acid intake, especially towards end of gestation. Thus, when considering a limited supply of feed to restrict energy consumption, it is important to provide a diet that allows a high efficiency in the utilization of the protein. Amino acids are not only building blocks of protein synthesis but are also used as precursors for nitrogenous substances essential for whole-body homeostasis (Wu et al., 2010). There is strong evidence that the members of the arginine family of amino acids have an important role in placental vascularization and development, especially during the middle of pregnancy (Wu et al., 2007). This theory is supported by Mateo et al. (2007) who supplemented 1% L-arginine to a corn and soybean meal-based diet after day 30 of gestation to gilts and found that supplementation increased the number of pigs born alive by 22%. There is more information available on lysine requirements than other essential amino acids. However, if we apply the ratio that makes ideal use of the proposed need of lysine by Samuel et al. (2008a), the need for methionine would be 40% higher than current recommendations. Methionine plays a key role during pregnancy, including DNA methylation during development of pregnancy, which makes it extremely important for the regulation of gene expression. Dourmad and Etienne (2002) concluded that the need for threonine during pregnancy in modern sows is greater than the value proposed by most published references on sow's requirements, the authors attribute this difference to a greater daily nitrogen retention found in modern sows during gestation. These results corroborate the hypothesis that the amino acid requirements are larger for current genotypes, due to a greater lean tissue deposition and protein turnover capacity. For foetal growth and development of mammary tissue to occur rapidly during the final stage of gestation, the amino acid requirements tend to be higher. Therefore, muscle growth must also be considered in younger sows as part of their reproductive needs. While analysing recent studies with modern genotype sows, particular attention has been given to foetal growth (McPherson et al., 2004) and development of the mammary gland (Ji et al., 2006). The results obtained by these authors indicate an exponential growth of both the foetus tissue as well as the mammary gland mainly after 70 days of gestation. These results are higher than those observed in similar studies in the '80s and the '90s. Wu et al. (1999) assessed the amino acid composition of pig fetuses during the different stages of gestation and observed that this variable changes significantly during the progress of gestation. The changes in the

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rate and composition of the tissue gain affect the individual needs for amino acids for fetal and mammary gland growth during gestation. From the amino acid composition of various tissues (mammary gland, uterus, fetus, placenta) and the changes that occur during pregnancy, it is possible to develop models based on the productive profile of the modern genotypes, through which we can obtain the real daily nutrient requirements for sows. Based on the results of recent studies, the establishment of nutritional programs using more diets and no longer a single diet throughout the entire gestation period may have not only several benefits for the sows and foetal development (McPherson et al., 2004) but also reducing the excretion of total N and ammonia emissions, which may contribute to higher animal productivity and create a more sustainable environmental system (Clowes et al., 2003a).

The simplest method of meeting the increasing energy and amino acid requirements of sows during late gestation is increasing the level of feed supplied during late gestation. It is proposed that simply increasing feed intake better meets the increasing nutrient demands of the sow. NRC (2012) outlined a 400 g/day (about 20%) feed intake increase after day 90 of gestation based on energy requirements, while this increase should be about 40% based on lysine requirements. A cooperative research study by Cromwell et al. (1989) concluded that additional feed supplied during late gestation improved reproductive performance. The study involved 1,080 litters where multiparous sows in the treatment group were fed 1.82 kg/d of a corn and soybean meal-based diet (3.2 Mcal ME, 14% CP) in addition to the levels received by the control group (summer 1.82, winter 2.27 kg/d) from day 90 of gestation until farrowing. Sows fed extra feed in late gestation farrowed an average of 0.35 more pigs/litter, as well as slightly heavier pigs at birth (1.48 kg vs 1.44 kg) and at weaning (18 days) (Cromwell et al., 1989). A more recent study by Shelton et al. (2003) yielded slightly conflicting results when 0.9 kg/d of extra feed (corn/soybean meal-based diet containing 3.26 Mcal ME, 0.57% SID lysine) was given after day 90 of gestation (2.09 vs 2.95 kg/d,). These authors found that increasing feed intake during late gestation led to a decrease in piglet birth weight in multiparous sows, but an increase in piglet birth weight in gilts. Additionally, gilts offered extra feed had an increase in subsequent conception rate compared to the control, whereas sows fed extra feed had reduced conception rate in subsequent parities. Only in second parity sows did an increased feeding level during late gestation slightly increase litter weight at weaning. We also have to consider that extra feed supplied during late gestation can result in over-conditioning of sows at farrowing, which can compromise sow reproductive performance (Young et al., 1991; NRC, 2012). Research focusing on relating patterns of intake of total feed, energy or protein (i.e., amino acids) during gestation to sow reproductive performance has yielded varying results. In an extensive review of the scientific literature, Campos et al. (2012) reported that providing extra feed or energy during late gestation only marginally improved piglet birth weight, and effects were not consistent between different studies. Several studies demonstrated no effect, while others indicated improvements in various aspects of production, such as litter size, gestation sow BW gain, lactation sow BW loss and feed intake during lactation. Differences in results amongst these studies could be attributed to different levels of energy and nutrients supplied, as well as different durations of time and periods of supplementation. Another important factor to consider is the use of primiparous sows compared to multiparous sows, which are known to have differences in nutrient partitioning. Current commercial gestation sow feeding strategies do not consider the sow as an individual; they are generally based on using a single gestation diet for all sows regardless of parity or stage of gestation. Computer controlled electronic sow feeders (AIPF) allow precision feeding (PF) of individual, gestating sows according to parity order and gestation stage housed in groups. Based on the above observations, it is clear that increasing dietary amino acid levels is more beneficial than increasing feed intake, especially during late gestation, as it does not contribute to excess maternal body lipid deposition which may reduce subsequent sow reproductive performance. While studies have clearly demonstrated that the amino acid demands of gestating sows change throughout gestation, more research is needed to clarify if more closely meeting these changing amino acid and energy requirements will improve sow reproductive performance and ultimately, profitability.

Use of AIPF (artificial intelligent precision feeding) Technology

The welfare of farm animals is important for producers, consumers and society as a whole (CORNISH et al., 2016). Modern hyperprolific sows are often fed restrictively for efficient reproduction and to increase longevity (MANU, 2020) and/or are fed only once a day, for reasons such as reduced farm management or the feeding system. Gestating sows that are fed restrictively may experience stress and impact on their behavior (BERNARDINO et al.,2016). Increased and sustained stress is associated with impaired well-being. The hypothalamic-pituitary-adrenal (HPA) axis is one of the physiological systems almost always activated by stress. In research carried out, it was possible to observe that the availability of food stimulates the rhythmicity of cortisol in such a way that food restriction or starvation increases the average levels of glucocorticoids in humans and rats (GARCIA-BELENGUER et al., 1993; KENNY et al., 2014). Cortisol is a steroid hormone secreted by the adrenal gland and has a circadian rhythm with highest concentration around 8:30 am, gradually decreasing to the lowest levels around midnight (CHAN and DEBONO, 2010; SUNAINA et al., 2016). In studies carried out by Amdi (2013), it was possible to observe that food restriction in pregnant gilts elicited higher levels of salivary cortisol than gilts used in the control treatment, which had higher feeding levels. When an animal is pregnant, the prolonged stress response, the hyperactivation of the HPA axis and the excess of glucocorticoids pose risks to normal development, reproduction, emotional balance, physiological health and the well-being of newborns (COULON et al. al., 2013; PETIT et al., 2015).

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With regard to feeding frequency, Verdon et al. (2018) found that increasing feeding frequency allowed the performance of natural behavior to improve welfare compared to less frequently fed sows. According to Meunier-Salaün et al., (2001), pregnant females are fed about 2.5 kg of feed per day, which represents 50% of their *ad libitum* feed intake. Dividing limited feeding into two or three meals or feeding these animals several times a day did not change basal cortisol concentrations, which is consistent with findings from other studies (TERPSTRA et al., 1978; LEVAY et al., 2010). In view of this, according to Manu (2020), in his study it was not expected to find any difference in basal cortisol levels when all treatment groups had similar energy intake per kilogram of metabolic body weight. However, it was observed that twice-daily feeding reduced the area under the curve (AUC) of cortisol compared to control sows. Farmer et al. (2002) also reported that feeding pregnant sows a concentrate diet twice daily reduced cortisol AUC compared to single-feeding sows.

Increasing feeding frequency for pregnant females can improve satiety and their well-being because energy for stereotyped behaviors can decrease and increase productivity. The increase in activity can be attributed to inadequate intestinal filling due to the lower amount of energy and/or volume of food received in each feeding. In support of this theory, Lawrence et al. (1988) explained that the conventional diet of North American sows is concentrated in nutrients and, although it is sufficient for good health and performance, it may not meet the animal's other needs. In addition, the small amount of food is unlikely to give a feeling of satiety (VERDON et al., 2018).

When the amount of the meal is too small to induce satiety, "non-eating activities" persist (TERLOUW et al., 1993; ROBERT et al., 2002). The behavioral activities that precede feed provision are termed "food anticipatory activity" (FAA) (JOHNSTON, 2014). A balanced diet guarantees adequate nutrients for each phase, but this is not synonymous with satiety of the sows, and this lack of satiety may reflect on stress and behavior (MEUNIER-SALAÜN et al., 2001; DE LEEUW et al., 2004). The fact that sows are hungry reflects in abnormal behaviors, arising from the absence of satiety and the presence of motivation to feed. This motivation is represented by behaviors such as rubbing the muzzle on the empty feeder and biting bars in the cell (DOUGLAS, 1998; JENSEN, 1980). Furthermore, these changes in motivation can alter the performance, the immune function and also the behavior of these animals, factors of extreme importance in productivity, economic viability and longevity of the sow (DOUGLAS et al., 1998).

According to Samuel (2019), there is a trio of "rights" in the precise feeding of modern and hyperprolific sows: the right diet, in the right amount and at the right time. Feeding levels for sows during pregnancy are normally based on maintenance requirements, desired body condition, weight and litter weight gains (NATIONAL RESEARCH COUNCIL, 1998; SPOOLDER and VERMEER, 2015; BUNTER et al., 2018). However, the amount of food is normally less than the amount that sows would voluntarily consume (VAN BARNEVELD et al., 2007). In electronic feeding systems, pregnant sows are usually fed only once a day, which has been shown to increase the efficiency of energy use, however, it has reduced the efficiency of protein use and, unfortunately, we still observe a scarcity of information. recent studies on feeding sows at the right time (SAMUEL, 2019). Previous research has not shown significant productivity and performance advantages when feeding sows more than once a day. According to Fabry (1969), feeding sows once a day seems to result in greater energy storage efficiency. The improvement in energy utilization efficiency was also attributed to reduced energy expenditure related to the consumption of a single meal compared to several meals (Baird, 1970). On the other hand, it was possible to observe that the reduction of feeding frequency has an effect on lipid metabolism. As an adaptive mechanism for storing large energy intakes, lipogenesis is stimulated by infrequent feeding of meals (LEVEILLE and HANSON, 1965). The result of increased lipogenesis is an increase in body fat and plasma lipid concentrations (FABRY, 1969). While animals fed a single meal tend to retain excess energy primarily as fat, animals fed more frequently tend to store excess carbohydrates as glycogen rather than converting them to lipids (LEVEILLE and HANSON, 1965). As a result, we can predict the composition of body weight gain among animals fed more frequently compared to those fed a single meal. Therefore, the effect of feeding on energy metabolism must be considered, mainly due to its impact on dietary needs and body composition of sows. Samuel (2019), investigating the energy and protein metabolism of pregnant and non-pregnant females (through simultaneous energy measurements using open circuit calorimetry and protein metabolism as protein turnover), showed that the frequency of feeding has a opposite impact on female metabolism. Therefore, while single-feeding improved energy retention efficiency, protein utilization efficiency was reduced (SAMUEL, 2008). More recently, Manu et al. (2019) reported that when sows fed in the afternoon instead of being fed in the morning, should changes in energy and nutrient metabolism by increasing backfat thickness.

Currently, a very important goal within the swine industry is to achieve precision feeding. One of the objectives in this regard is to reduce the crude protein content of the swine diet. Potential advantages of low crude protein diets include savings on expensive protein ingredients, reducing dietary costs, lowering nitrogen emissions, lowering the impact on the environment, thus improving gut health and production efficiency. Probably, dietary requirements for amino acids are higher due to single-meal feeding, where protein utilization is reduced (SAMUEL, 2019). In addition, reductions in the crude protein content of the diet will require higher levels and mixtures of synthetic amino acids in the diets. Therefore, the potential for infrequent feeding can negatively impact amino acid utilization due to nutrient asynchrony. Amino acid asynchrony refers to the delayed digestion and absorption of protein-bound amino acids

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compared to synthetic amino acid sources. However, there may be potential implications for increasing the inclusion of synthetic amino acids, such as in low crude protein and single-meal diets.

Using AIPF technology can accommodate the unique and dynamic changes in energy and amino acid requirements of individual sows throughout gestation. Still there are challenges concerning the implementation of this technology. The limitations of the application of a production system in a commercial farm are not only related to theory (making mathematical models), but the work to be performed with the animals must be treated with great care. In addition, the adaptations in the facilities for the application of this system, as well as the acquisition of such technologies, can impact on production costs if not properly managed.

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Attributing *Toxoplasma gondii* infections to sources: currentknowledge and addressing data gaps

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Introduction

Toxoplasmosis is an important foodborne disease worldwide. Its public health importance has been largely under-recognized, but recent evidence has shown that *Toxoplasma gondii* leads to a high burden of disease at global, regional and national level. The World Health Organization ranked toxoplasmosis as number 13th among 31 foodborne diseases globally, also demonstrating regional differences, with for example a relative higher importance of toxoplasmosis in the Americas than in Europe (WHO, 2015). Several other studies have estimated a high public health impact in several regions (Torgerson et al., 2015) and specific countries (see. e.g. (Havelaar et al., 2007; Nissen et al., 2017; Scallan et al., 2015; Van Lier et al., 2016)).

Humans can be infected by *T. gondii* post-natal (i.e. acquired toxoplasmosis) or vertically (i.e. congenital toxoplasmosis). Because congenital toxoplasmosis is considered particularly problematic due to the severe health effects it can cause in children since birth and the possibility of fetal death, its public health impact has been more extensively studied than acquired toxoplasmosis, which is usually associated with mild flu-like symptoms. However, several newer studies suggest that in some cases ocular disease and severe syndromes such as psychiatric disorders, as well as suicide attempts and traffic accidents, may develop asa result of infection (Burgdorf et al., 2019; Flegr et al., 2014). Furthermore, infections acquired post-natal can cause ocular disease, and atypical strains, which are common in areas outside of Europe, have caused severe toxoplasmosis even in immunocompetent individuals.

Cats and wild felids are the only definite hosts of the parasite, but virtually all warm-blooded animals can act as intermediate hosts, and most species can be carriers of tissue cysts of *T. gondii*. *T. gondii* has been isolated from most livestock species such as pigs, cattle, sheep, poultry, as well as wildlife and game. Like many other foodborne hazards, *T. gondii* can be transmitted to humans through consumption of contaminated foods but also by other routes: through water, soil, or air; by direct contact between people, or by contact between people and animals. The relative importance of exposure from a contaminated environment versus consumption of meat or other foods is still unclear.

To identify and prioritize interventions for reducing the burden of foodborne diseases, evidence on the relative contribution of different sources and routes of transmission of *T. gondii* at regional and nationallevels is needed.

Source attribution of foodborne diseases

The process of partitioning the human disease burden of a foodborne infection to specific sources is known as *source attribution*, where the term source includes reservoirs (e.g. animal reservoirs like pigs, cattle, pets) and vehicles (e.g. food products like pork or beef) (Pires et al., 2009). Source attribution studies may also be able to distinguish between the contribution of different transmission routes from one or more sources for infection. A variety of methods to attribute foodborne diseases to sources are available, including approaches based on analysis of data of occurrence of the pathogen in sources and humans, epidemiological studies, intervention studies, and expert elicitations. Each of these methods presents advantages and limitations, and the usefulness of each depends on the public health questions being addressed and on the characteristics and distribution of the hazard (Pires, 2013).

Source attribution methods have been extensively used to investigate the contribution of food and animal sources for several infectious diseases, e.g. salmonellosis, campylobacteriosis, and listeriosis. Measuring the proportion of foodborne infections that is attributable to different sources has proven useful in several countries and regions, contributing to *One Health* efforts to guide food-safety interventions based on scientific evidence. However, application of source attribution methods for several zoonotic pathogens is often more challenging, which can be due to the characteristics of the pathogen or due to lack of data. To a large extent, this has been the case of *T. gondii*, for which robust, data-driven source attribution studies in most countries are still lacking.

Overview of studies attributing *T. gondii* infections to sources

Source attribution of toxoplasmosis is particularly challenging due to lack of data, and few studies have been

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conducted so far. To overcome this challenge, WHO's Initiative to estimate the global burden of foodborne diseases included a large expert elicitation study to assess the contribution of sources for several diseases, including toxoplasmosis. This study estimated that between 42 and 61% of acquired toxoplasmosis cases globally are due to foodborne transmission, with other important routes being water (11-27%) and soil (18-38%) (Hald et al., 2016). The next step of the source attribution process is to measure the contribution of specific sources within these major transmission routes, which would ideally be based on data on prevalence, contamination and exposure of/to each source.

Opsteegh et al. measured the relative contribution of three meat types for infection with *T. gondii* in the Netherlands (Opsteegh et al., 2011). The authors used a comparative risk assessment approach and concluded that 70% of meat-related infections were due to consumption of beef products, 14% due to sheep meat, and that 11% were attributable to pork products. A more recent study from the same group has improved the model to include the effect of salting on parasite viability, as well as lower concentrations of bradyzoites in cattle, more specific heating profiles, and more recent consumption data (Deng et al., 2020). Results showed that beef remains the most important source of *T. gondii* in the Netherlands, contributing to 84% of the total number of predicted infections in the Dutch population, followed by pork (12%), mutton (3.7%), lamb (0.2%) pork/beef mixed products (0.1%), and veal (0.01%) (Deng et al., 2020). Quantitative risk assessment studies of *T. gondii* in meat products have also been conducted in the UK, Italy, the United States (Condoleo et al., 2018; Crotta et al., 2017; Guo et al., 2017). Guo et al. (2015) performed a qualitative risk assessment of meatborne toxoplasmosis in the United States, and estimated that exposure associated with meats from free-range chickens, and non-confinement-raised pigs, goats, and lamb were higher than those from caged chickens and confinement-raised pigs and cattle (Guo et al., 2015). Belluco et al. (2018) compared the relative risk of *T. gondii* exposure through bovine meat vs pork in Italy, and found that bovine meat was found to be a more likely route of transmission to consumers than pork (Belluco et al., 2018). Condoleo et al. (2018) estimated the risk associated with consumption of different pork products in Italy, and concluded that almost all infections are associatedwith the consumption of fresh meat cuts and preparations, and only a small percentage is due to fermented sausages/salami (Condoleo et al., 2018). Remaining risk assessment studies developed models that will be useful to inform source attribution models, but looked only into one type of food/ transmission route, and thus do not provide evidence on the relative contribution of sources for infections.

A case-control study in the United States found that the leading foodborne risks associated with toxoplasmosis were eating raw ground beef, rare lamb or processed meats produced and consumed without heat treatment (Jones et al., 2009). More recently, a systematic review and meta-analysis of case-control studies supported these estimates by identifying three risk factors of toxoplasmosis: consumption of raw/ undercooked meat, consumption of raw/undercooked beef, and consumption of raw/undercooked sheep meat (Belluco et al., 2018).

In the absence of data for the application of data-driven methods as described above for a regional and global study, the expert elicitation conducted by the WHO described above also measured the proportion of foodborne *T. gondii* infections attributable to specific foods (Hoffmann et al., 2017). In this study, red meats (i.e. beef, small ruminants' meat and pork) were estimated to cause 50% to 64% of foodborne cases in all regions, but the specific source of that exposure was estimated to vary markedly across sub-regions. Small ruminants' meat was estimated to cause over 40% of foodborne toxoplasmosis in the Eastern Mediterranean Regions, while beef was estimated to cause 30% to 40% in Africa and several sub-regions of the Americas, Europe and Western Pacific. Pork was estimated to account for roughly 20% of foodborne toxoplasmosis in less developed regions of the Americas, and in developed countries within Europe. Vegetables were estimated to play a slightly larger role (21% to 23%) in Europe and South-East Asia than in other sub-regions (14% to 19%). Eggs and dairy are not believed to contribute to foodborne toxoplasmosis.

To our knowledge, these are the published studies on source attribution of toxoplasmosis. Because the geographical differences in the epidemiology of *T. gondii* as well as in consumption habits affect the relative importance of different sources, results from few local studies cannot be extrapolated to other countries.

Main challenges and data gaps

Even though several studies investigated the sero-prevalence of *T. gondii* in different sources, including animals and foods, representative data from all potential sources of the parasite are still lacking globally. Such data are essential for estimating the relative contribution of each source. Furthermore, the variety routes of transmission of the parasite, which include consumption of contaminated meat products, but also other foods, contact with live animals and environmental exposure, make the data requirements and modelling exercises particularly demanding.

The comparative exposure assessment appears to be the best approach to perform source attribution. However, this is a *data-hungry* method, relying on representative data on source contamination, exposure and effect of different processing steps in the survival of the parasite through the transmission chain. The risk assessment studies that have

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been developed to estimate the risk of disease through consumption of one specific food type can be used as a baseline for further method development. Still to address are substantial data gaps.

What are we doing to address knowledge gaps?

A pan-European project – "TOXOSOURCES: Toxoplasma gondii sources quantified" (2019-2023) - is collecting and analysing data to identify and rank the most important sources of *T. gondii* in the region[1]. In this regional initiative, raw data and published data are being collected for countries across Europe, enabling an estimation of the relative contribution of the different sources for infection. To inform source attribution in the region, TOXOSURCES will estimate the relative contribution of food and environmental transmission routes, provide an overview of the prevalence in food animals and cats, quantify human exposure to possible sources of infection, provide an overview of the processing parameters for relevant meat products, and provide an overview of prevalence and risk factors of human infection. The project will develop a methodological framework and deliver evidence for risk management, including prioritization of food safety strategies. We expect that this approach will be useful derive national and regional source attribution estimates for toxoplasmosis, identify differences between countries, and help understanding the reasons for such differences. Furthermore, the framework may be useful to apply similar studies in other countries and regions.

At the global level, the WHO reactivated the Foodborne Disease Burden Epidemiology Reference Group (FERG) in 2021 to update the global and regional estimates of the burden of foodborne diseases. The initiative will also update source attribution estimates for all foodborne hazards included, namely T. gondii. Results are expected by 2025.

Conclusions

Conducting source attribution of toxoplasmosis has been challenging globally due to substantial data gaps. This is true even for countries with extensive and well-established surveillance and monitoring of foodborne diseases in foods, animals and humans. The evidence compiled so far points to a high contribution of beef and ruminant meat for foodborne infections globally. Lower attribution proportions have been estimated for pork products by some studies, and raw or undercooked vegetables may also be relevant sources of toxoplasmosis. However, substantial data gaps remain. Importantly, estimating the relative contribution ofnon-foodborne transmission routes, such as contact with animals or environmental transmission, is crucial to inform public health policies. Secondly, developing and applying more robust data-driven methods, suchas comparative exposure assessments and national or regional level, requires collecting representative and comparative data from multiple sources, including source contamination, exposure, survival and dose-response data. We expect that recently launched large scale projects will provide a unique opportunity to address these knowledge gaps, and anticipate that upcoming research will focus on toxoplasmosis and further expand these efforts

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Past, present and future of reproduction in the pig breeding herd

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Introduction

The swine industry has seen numerous changes occur in the housing and reproductive management of the breeding herd over the last 30 years. Rather than review the history of that evolution, it is perhaps more interesting and relevant to identify what factors have had the greatest impact on the acceleration and direction of changes in breeding herd production practices. While the location and size of the pig industries around the world has not shown dramatic changes over the last decades, it has fluctuated the most in response to global market access and animal disease outbreaks. But changes in breeding herd numbers and industry structure have also occurred in response to export markets and monetary exchange rates, political agreements for trade, animal welfare and food safety regulations, production capacity, and size of the domestic and international market for pork. The major challenges and opportunities in the future for swine breeding herds will involve their ability to remain competitive through production efficiency, animal welfare, herd health, reduced environmental impact, and retaining qualified labor. While the industry continues to adapt and adjust to these issues from previous years, others are now becoming more prevalent and may alter and accelerate the direction of change for how the breeding herds will operate in the future. This article will briefly address some of the major advancements that have occurred in production practices on breeding farms as a result of discoveries in swine reproductive sciences. The major sow populations for modern commercial breeding herds located in China, Europe, the Americas, and in other parts of Asia, are similar in many ways, but with nuances for particular regions. In each of these areas, industry consolidation continues, with the number of farms declining and the number of sows owned by a single operation increasing. Financial ownership and control of these large farmsis also more complex, and not necessarily restricted to the country where production is located. Nevertheless, this trend for the breeding farms continues with continued emphasis on making improvements in production efficiency, animal health and welfare, food safety and pork quality.

Reproductive management with disease

Over the last 30 years, the swine industry has been challenged by the emergence and spread of viral diseases of global importance, with the greatest concern for ASF, followed by PEDV, and the ongoing challenge for farms dealing with PRRS. Each of these diseases has resulted in significant mortality within the breeding herds. And while outbreaks of ASF require depopulation, the outbreaks with PEDV and PRRS, while causing high mortality and morbidity, allow management through disease outbreaks. Both PRRS and PEDV have been interesting, as producers have attempted to stabilize herd health while continuing with breeding herd production flow. Over decades, PRRS has driven the development of many of the biosecurity procedures in use today, for how breeding herds operate and manage animal flow. For PRRS, it has been shown that the virus can be transferred onto a farm from animals, people, and vehicles. Because of this, biosecurity procedures have been widely implemented to control their entry. But what has also been interesting, is that in cases where fertility of the sows and survival of the piglets has been impacted, there have been approaches to delay or synchronize estrus and re-establish breeding groups from sows that have lost or had all their removed due to disease. The use of progestogen has been implemented in these early wean scenarios, with some data available on its use after the first week (dos Santos et al., 2004). But little formalized research has been performed as the pig losses can often occur within the first week after farrowing and under disease outbreak scenarios, there is little opportunity for pre-planned design. Nevertheless, farms experiencing PRRS or PEDV outbreaks with expectations for mortality in the piglets, have implemented an estrus suppression/synchronization protocol by feeding progestogenin the first weeks following farrowing and piglet loss. These farms have been successful at re-establishing the breeding groups but do not allow breeding until the females reach 2 weeks post farrowing. This makes sense as the function of the hypothalamic-pituitary axis and uterine repair are typically not restored until 2-3 weeks following parturition.

Modern genotypes

The use of modern swine genotypes allows farms the capability to efficiently produce a large litter of pigs. Further, these pigs are genetically designed to be fast growing and feed efficient, and when they reach market weight, produce carcasses with a high yield of lean meat. Nowadays, only a few breeds of pigs are used in the production of commercial pork around the world. In fact, there is limited genetic diversity in the foundation breeds used by commercial producers. This of course, will make selection to minimize inbreeding an even greater challenge in the future. The

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foundation maternal pure breeds in most nucleus farms are based on Large White and Landrace heritage, while the terminal line foundations most often involve Duroc or Pietran breeds. As with commercial production farms, consolidation continues in genetic suppliers, and this will continue to limit diversity. However, as the selection methods continue to advance, opportunities can be created with new lines. In the future, the opportunities for the breeding farms to create and capture heterogeneity in both the F1 and F2 females may eventually require specified matings from single sires and not from a mixture of semen from multiple sires. The modern female pig is genetically selected for fast, lean growth, high yield carcasses, larger litter size, and a high number of functional teats. During development, many of these females now grow very fast, with a high probability that some will be too heavy by the time they express puberty and are mated. This will have detrimental effects on their structure in their 1st and 2nd parities and will likely lead to reduced productivity and longevity. To date, answers for how to slow down their growth without reducing fertility, is still unclear. To do this effectively, farms will need a way to obtain gilt weight, and an approach for how to adjust feeding for these gilts. It is also not clear whether the fastest growing gilts might be able to be induced into puberty earlier in order to target breeding at an optimal weight at 2nd estrus. Information would be needed to determine whether this is a good approach for fats growing females for lifetime retention and fertility. There has been much interest throughout the years on the effect of increasing leanness and reduced body fat on puberty and fertility in gilts. Whether these trends will continue in the future is not clear, but breeders and producers are concerned about the detrimental impact of leanness in the replacement females, and while problems are suspected, only limited associations with delayed puberty have been identified. But simply looking only at gilt leanness, does not allow focus on potential problems when they reach their first lactation. This is the phase when animals with limited body fat stores may be unable to mobilize the resources needed for milk production without significant catabolism of muscle.

In addition, increased litter size has been clearly trending up each year as a result of improvements in genetic selection, male and female fertility management, estrous detection and AI, as well as improvements in animal housing, feeding and health. The trends and increases have been evident since the early 1990s, and the clear slow linear progression continues on today. The question is will this trend continue on into the future, and is this a good or a bad pathway. The increase in litter size results directly from an increased ovulation rate for numbers of eggs, and an increase in uterine capacity. But at the same time, there has also been a notable decrease in average litter birthweight, and in some cases, a greater spread within litter birthweights, or a greater frequency of low birthweight piglets (Quesnel et al., 2008). This has now created the scenario where the smaller pigs in the litter are less likely to survive. And if they do survive they are at greater risk for slower growth, reduced feed efficiency, poor health, and delayed age at puberty (Almeida et al., 2017; Patterson et al., 2020). While the lowest birthweight pigs, < 1.0 kg typically are poor performers in all categories and should not be selected as replacements (Magnabosco et al., 2016), the selection of the larger piglets is sometimes not as clear. Perhaps the relevant point is that if the piglets are born heavy in a large litter size, this would be very good, but heavy piglets in a small birth litter should not be selected. Further, the data now implicates low birthweight litters as a repeatable phenotype and suggests gilts from these litters should not be selected as future replacements. Although the information is speculative, with the increase in litter size and selection of females for more functional teats, there are some indications that the length of the female has increased. This is loosely based onfemale size observations in fixed length farrowing crates. If true, this will require a change in the dimensions for the housing crate in farrowing.

Reproduction metrics, parity and season

The measures used for assessing the performance in modern swine production systems can often be reduced to a single measure or a long list of measures. Single measures such as pigs produced per sow per year certainly capture several different fertility aspects at once. However, other measures are clearly needed and informative and can be important for consideration as to whether the individual animal or the herd are reaching their fertility potential. The goal for farms to reach very high reproductive rates is a reality today, and the females on these farms can be characterized as being hyperprolific (Kemp and Soede, 2012b). Whether this female represents a genetic population, or a sub-set of the larger group is uncertain, but clearly it displays the fertility potential at the upper range. Even in the hyperprolific female population, there is an expected normal distribution for measures such as litter size. But it is likely that the ability of the modern genotype sow to reach the hyperprolific state as an individual or as a herd is perhaps more dependent upon farm management, than genetic limits. In fact, notable differences separate farms on sow reproductive performance (PigChamp, 2022). Top performing farms are above average in multiple categories and the hyperprolific sow would be expected to represent the majority of females in that farm population, while on lower performing farms, these animals would be limited in their genetic potential (Knox, 2022). What is not entirely clear is which modern genotypes in commercial production have the potential to reach hyper-prolificacy or whether environmental influences are restrictive to this state. Nevertheless, a hyperprolific sow would need to be in the highest categories for total pigs born, pigs born alive, litters per year, and bred within 7 days of weaning. There is now an expectation that 90% of weaned sows will be mated within 6 d of weaning, 90% pregnant at day 30 and that farrowing rates will reach >87%. The total born litter size is now expected at 14.5-16.0 pigs. It is also notable that gestation lengths now average 115-116 days from service, based on farm records, and may or may not be related to the increase in litter size. Perhaps also

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worthy of mention is whether older data regarding the effects of parity are still true today. There have been numerous reports over decades, including the present day, indicating that parity 1 and 2 sows may be more susceptible to reproductive failures that older sows (Hoving et al., 2010). This can be substantiated for Parity 1 sows, but the 2nd parity decline in litter size is not as clear. In fact, for either animal, the likelihood of failure may be related more to gilt development, and management during first gestation and lactation, than any inherent failure in that parity. It is clear, when examining the fertility rates for parity 1 sows, many animals and herds are quite capable of managing those females as a hyperprolific sow. But environmental influences, such as heat stress, may interact with the development of these young females and put them at greater risk for reproductive failures or sub-standard performance. Seasonal declines in fertility are notable in pigs around the world. These periods of reduced fertility may be related to heat stress resulting from high temperature and humidity in summer or related to photoperiod sensitivity, especially during long days or periods where photoperiod is transitioning to shorter days. The effects have been documented in boars with increases in sperm abnormalities occurring in certain seasons such as summer or fall. Fertility declines may also be evident in the sow breeding herds with notable effects in parity 1 sows for delays in estrus after weaning and reduced conception rates. The seasonal effects are clearly quite diverse around the globe. Even within the same area, herds with similar genetics and management display differential susceptibility and fertility symptoms to season and by parity. This observation suggests that farm management plays a major role in the impact of seasonality on breeding herd fertility.

Housing the breeding herd

Around the world, dramatic changes in the housing of pigs in the breeding are occurring, whether required by regulations or for decisions related to market access or company philosophy. For the breeding herds, this involves the ability to house male or female breeding pigs in individual crates continuously, for a limited period of time, or at all. In many parts of the world, there are no welfare restrictions and the majority of the sows and boars in the breeding herd are housed in crates in some form of environmentally regulated buildings. In other countries around the world, the use of crates is restricted to a few days around breeding and for farrowing. Production at very high efficiency has been reached regardless of housing in pens or crates. But while management in crates is very similar around the world, there are numerous differences in management in the pen and loose housing systems. The diversity in options for housing and managing sows in groups are numerous and can be related to farm size, facility costs, labor, fertility and welfare. Sows that are weaned into groups can be stimulated and detected for estrus, inseminated, establish pregnancy and farrow a large litter, but there are greater challenges in these systems that require new management approaches (Kemp and Soede, 2012a). The opportunities for the future of group housing are discovery of what approaches provide a practical and efficient way for farms to reach the fertility and welfare goals when sows are in static or dynamic groups, and in manual or electronic management systems (Knox et al., 2014). Because there are numerous combinations for housing and feeding options for the breeding herd, each farm will need to determine what procedures work best for reproductive management for achieving fertility and welfare goals (Bracke et al., 2002). It is likely, that new technology related to electronic animal identification and monitoring, will continue to increasing role in the future.

Induction and detection of fertility in pigs

The stimulation and detection of puberty in the gilt is an important component to success in the breeding herd. The gilt requires a considerable investment of resources during development to reach maturation, in order to express pubertal estrus at a targeted age and weight. Problems arise because when the female reaches an appropriate age, a continuous effort for puberty stimulation and estrus detection is required, which can extend for months, especially when there are delayed and non-responders in the gilt pool. In many modern systems, where replacement gilts are managed in batches to minimize biosecurity related entry events, cohorts of gilts eventually move through a selection and stimulation phases as they age. In these groups, farms must be able recognize slow and fast growing gilts and devise a system for regulating their growth to prevent problems. Each farm will have specific procedures related to housing, gilt group size, and the age and method for application of boar stimulation. Most farms follow recommendations that induce puberty and then target breeding the gilts on their second estrus provided the gilts have reached an appropriate age, and weight. In many of the electronic feeding systems in use for group housed sows, training gilts for use of these systems is required before they enter into the breeding groups. During the training phase, they need to learn to move through the electronic gates, then how to open the gates, and lastly how to access their feed.

Weaned sows comprise the majority of the breeding herd and individual breeding groups and therefore the weaning event sets the timing for flow of pigs (Knox, 2019). Over the decades, there have been changes in lactation length on farms. Nowadays, farms with animal welfare regulations may be required to allow ~ 4 weeks before weaning while in other parts of the world, lactation length is ~21 days. There is a clear trend for increasing age at weaning, and this

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likely reflects the benefits for the fertility of the sow and the health and weight of the piglets. The longer lactation lengths are also related to the higher litter size and farrowing rates observed in herds today, when compared to shorter periods. At one time, early weaning at 12-14 days, was used for prevention of disease transmission to piglets, but this approach was detrimental to sow fertility and pigs did not grow as well as expected. Lactation requires large energy and protein contributions from the sow and she must consume adequate amounts of feed each day to produce high amounts of milk. When the sow is not able to consume enough feed, she will mobilize body reserves, and may eventually fail to reach her milk production potential. Certain farm management procedures have been used to allow sows to retain more of their body condition during periods of high milk production. These practices have involved partial or split weaning of the heavier piglets from the litter in later lactation. Weaning age also has a direct impact of the wean to estrus interval in sows. The optimal interval to estrus is 4-5 days following weaning and should represent ~90% of the sows. But variation in this measure occurs, and some fertile sows display estrus as early as day 3 or later on day 6. Estrus expression on day 2 or ≥7 days post weaning would be considered abnormal, and in either case would warrant investigation into the source. Accelerated rates of follicle development might suggest nursing may have terminated earlier or that animals are starting to cycle during a longer lactation. Delays in wean to estrus intervals are nearly always related to slow follicle development, which is most often caused by factors involving negative energy balance and poor body condition at weaning in young parity females in certain seasons of the year.

Hormone control of reproduction

The hormones that are available for use in controlling reproduction have not changed dramatically over the years and are somewhat limiting. But new formulations and discoveries continue and could change their use in the future. For the past 30 years, equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) have been widely used for stimulating follicle development and ovulation in prepubertal gilts or in weaned sows. The injection of the glycoprotein hormones in the proper doses stimulates follicle selection and rapid development towards ovulation within 5 days. But problems in hormone production, sourcing and availability are likely to be obstacles for use in the swine industry in the future. Further, while the effects for induction of follicle development with eCG or eCG+hCG (PG600) are generally good (80%), incidence of cysts (10%), and ovulation without expression of estrus (20%) can occur (Lewchalermwong et al., 2020). The development of alternative sources of hormones, perhaps using recombinant technologies, could work, but would need to go through extensive regulatory steps and even then, still might not be accepted by food production companies or consumers. However, other hormones and substances might have potential for use. Numerous GnRH analogues with enhanced or antagonistic activity have been developed (Brussow et al., 2009), and with some alternatives in their method for administration, such as in an injection-free, intravaginal gel (Ulguim et al., 2018). But while the agonists tested so far, have been effective for inducing an LH surge, they have not been successful at inducing sustained FSH release to stimulate follicle growth (Brüssow et al., 2010). To stimulate FSH synthesis and release might require an alternative approach using hormone combinations involving GnRH, ovarian proteins and estrogen, but this would be complex and not easy to implement. Perhaps some of the new neuropeptide regulators of GnRH would be more promising in the future. Kisspeptin for example, has been receiving much attention in multiple species, as an upstream neuropeptide that has been shown to be important for regulating GnRH and LH release (Lents, 2019). Further, the neural pathways and peptides identified in mice may also provide some insight into how these and other substances act to control reproductive cycles and sexual behaviors (Maeda et al., 2007; Hellier et al., 2018). One major obstacle for the swine industry has been the limited use of estrogen to control reproduction in the pig. There may be potential for its use in regulating control of the hypothalamus and pituitary axis, especially if practical administration and safety concerns could be reached. Over many decades, another steroid, the synthetic progestogen, altrenogest, has been approved and widely used for control of reproduction in pigs. Use of altrenogest has been effective for synchronizing estrus (Davis et al., 1985) and in farrowing (Gaggini et al., 2012). The hormone is administered as a top dress on the daily feed for 14-18 days for animals fed individually, while some have successfully transitioned to oral dosing individuals that are housed loosely in pens. Although perhaps not a major problem, concerns persist that some synchronization failures, or even abnormalities such as cysts, originate due to partial dosing or a missed altrenogest dosing day, regardless of whether the females are in stalls or in pens. In the future, perhaps new formulations or even alternative delivery methods could provide extended biological activity and reduce the need for individual daily dosing. Perhaps in electronic feeding systems, dosing will also become automated. Lastly, there has been an interesting increase in the use of synchronized farrowing in the last years. This may be related to the increase in gestation length previously noted, or to a shortage of labor, or the value in saving pigs. Many farms nowadays, choose to induce all females on day 114, or may only induce those that have not farrowing by d 116. Nevertheless, despite the increased trend for use, there has been little change in the hormones available for control of farrowing, which includes prostaglandin and oxytocin and their analogues. With the use of these hormones, in various sequences and timings, the same distributions occur, with only 40-60% of the females farrowing during the working day. This suggests there is still great opportunity to improve the results, and perhaps this could come with a change in hormone formulation, method of release or site of deposition.

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Breeding and AI approaches

The predominant method for identifying fertility in female pigs is through detection of estrus. This can occur while animals are housed in pens or crates and can employ fenceline or physical boar exposure. There are many variations for the process, which can depend upon how the animals are housed. But estrus detection is generally considered labor intensive as the boar or females must be moved in close approximation to each other while observing the female for the standing response. Because estrus symptoms can be influenced by environment, management, technical experience, and housing, variation in estrus detection often occurs. The boar is known to be the most important component of the stimulation process (Patterson et al., 2002). In fact, boar exposure on sequential days has been shown to improve and advance estrus in weaned sows and in gilts (Pearce and Pearce, 1992). The components of the boar that are known to be relevant for female stimulation and expression of estrus include his odor, vocalization, tactile pressure, and appearance (Signoret et al., 1975; Pearce and Hughes, 1987). Despite the fact that estrus is detected in a high proportion of sows after weaning, and in gilts expressing puberty, there is still considerable variation in its occurrence. In fact, 10-15% of sows may regularly fail to express estrus within 7 days of weaning. In gilts, onset of puberty extend over 2 months in the same batch. What could account for this extent of variation has been of great interest. In many farms today, the ratio of boars to females is extremely low, and the activity of boars exhibiting stimulatory vocalization, salivation, or interest in the females is limited. Further, eliciting the standing response is also highly dependent upon the application of the back-pressure test by the farm staff in the presence of the boar. This typically requires the technician apply hand pressure and rubbing on the back and flank of the females. This is physically demanding work, especially for large numbers of animals, and often the standing responses of the females can vary with some showing only weak symptoms. The different strengths of the estrus symptoms often require technicians spend more time with the females showing weak estrus and still can lead to uncertainty in classification for estrus. The problem for stimulation and detection of estrus, with this variation in symptoms, can become problematic, especially when considering many farms have limited labor and provide only a minimal time of exposure and limited physical stimuli.

Perhaps a potential solution in the future may involve the use of estrous stimulation and detection aids. In recent years, the development of various technologies has increased dramatically and some of these have been targeted for use in the pig breeding herd (Cornou, 2006). These can include recorded boar sounds, pheromones, and a robotic boar stimulation (Gerritsen et al., 2005). For aiding in advancing puberty and detection of estrus, synthetic pheromone products are available, with some new products on the market employing variations of the sub-maxillary C-19 steroids (McGlone et al., 2019). These products are administered to aerosolize for individuals or pens of females. However, for the pheromones, the scientific literature indicates that the estrus responses are often not as good as the boar, and can depend upon dose and combinations of the steroids used (Pearce and Hughes, 1987). The pheromones alone, boar recordings, and the robotic boar that utilizes all forms of stimulation, are still only partially effective in detecting estrus, and with only limited data on their ability to advance estrus. Perhaps what is needed is a better understanding of how the sequence for these stimuli are perceived by the female. Some females display the standing response simply being near a boar or even to back-pressure alone without a boar. However, the majority of females require additional time and stimulation. These behavioral responses are also known to change during the late follicle phase as estrogen levels change in blood (Langendijk et al., 2003). Because the pathways for gonadotropin release and expression of female behaviors are complex and likely controlled by different neural pathways, research into how the various stimuli affect these pathways may be needed to understand how to deliver and sequence artificial stimulation to achieve accurate results. Other tools such as robotics have been available for years to aid in moving boars, but there is the potential for robots to assist in the physical application for back-pressure and delivery of artificial boar stimulation in pens or stalls. What also may be of some value in the future is to develop tools that can help farms quantify the quality of the stimulation emitted by a selected boar for vocalization or for pheromone production. Technologies are available that can perhaps assess the level and compounds aerosolized by the boar and that are stimulatory to females. It may also be important to analyze the sequence, range and level of sounds emitted by the boar that are effective for stimulating females to express estrus. Electronic estrus detection systems are now in use in production operations around the world, for use in pens and stalls, and can identify females in estrus based on behaviors detected by various types of sensors (Bressers et al., 1991). Some equipment records animal behaviors or physiological measures with the use of video, thermal, motion, or proximity sensors. These data are included into an algorithm that has been used to identify estrus. While many of these are in use today and can clearly identify estrus and help achieve fertility targets, information is still needed on the overall accuracy for the detection systems and also for their level of contribution toward delivery of needed boar stimulation.

For many years now, the majority of commercial swine have been bred using artificial insemination with conventional AI rods (CAI), which deposits sperm in the cervix. More recently, intrauterine insemination (IUI, also known as PCAI, for post cervical artificial insemination) has been increasing, where semen is deposited in the uterine body (Knox, 2016). The fertility with both deposition sites is very good, and expertise can be developed with either. The advantages for PCAI include the potential to use fewer sperm per insemination in a smaller volume, and with less time required for insemination (Hernández-Caravaca et al., 2012; Sbardella et al., 2014). But PCAI does require more technical skill and training, and takes longer to insert the inner rod through the cervix. It can also pose some risk for sow discomfort

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and damage to the cervix, and the inner rod may not be passable in females with a smaller cervix, such as gilts and some parity 1 sows. Nevertheless, the trend is clear, and more operations are using the PCAI technology and with new developments, many of the catheter limitations may be overcome. Most sows are currently inseminated based on estrus with an insemination given on each day of standing heat, and most sows receiving two inseminations on average. But the incentive to accelerate genetic improvement by using sperm from higher indexing sires to breed more females has been an industry goal for some time. This could be accomplished by use of lower numbers of sperm in either a single or even a double insemination. The technology for use of a single AI requires synchronization of ovulation followed by a timed AI. This approach requires synchronization of follicle development, which can arise from use of altrenogest in gilts, or weaning in sows, and could also include the use of gonadotropins. At some later stage of the follicle phase, an ovulation induction hormone such as GnRH or its analogues, hCG, or LH can be given to synchronize ovulation. The technique works reasonably well, and some farms and studies can achieve similar fertility to those bred multiple times based on estrus (Brussow et al., 2009; Knox et al., 2017). However, because follicle development is often not optimal nor synchronous in the females that will receive a single AI, variation in the fertility responses have been noted (Knox et al., 2011). At the present time, this has limited adoption of the technology for a single timed AI, as pig production efficiency is of clearer economic value than greater genetic gain. Nevertheless, advances in diagnostics for assessing stage of follicle development and improved gonadotropin stimulation, could help select only fertile animals or prevent the occurrence of infertile animals assigned for this technology.

Boars and sperm production

Semen is routinely collected from high indexing boars housed in AI centers. Much has changed over the last 30 years in how boars are managed for semen production (Broekhuijse et al., 2011; Schulze et al., 2019; Waberski et al., 2019). In general, boars are kept on a weekly collection schedule, where the collection processes are defined to maximize sperm collection, sperm fertility, and to minimize bacterial contamination (Knox et al., 2008). Semen extenders classified as short, intermediate and long-term are still widely used to dilute and extend the fertile life of sperm while held at ~16 °C. Semen fertility is optimal when used within the guidelines for each extender. Nevertheless, most farms use semen within 2-4 days from collection regardless of the extender. New trends in semen processing have focused on eliminating the need for antibiotics, with use of new extender formulations that allow lower temperature storage, or new packaging materials that limit bacterial growth. The increased use of intrauterine insemination has reduced numbers, volume and package size for individual doses, and also facilitated the use of semen in bulk form for multiple dosing. Each of these has required changes in semen processing and packaging, and use on farm. And while advancements in AI processes can often result in good fertility, time is still required as farms adjust to changes.

Semen assessment and processing methodology used in the AI centers has probably shown the greatest extent of technology innovation and adoption (Didion, 2008; Feitsma et al., 2011). Semen processing procedures are well-defined and include an initial assessment for sperm motility, quality, and concentration (Schulze et al., 2015). Many larger AI centers around the world have been able to include the use of computer assisted semen analysis (CASA) systems to improve the speed and accuracy of semen evaluation and dose production. The high fertility in the swine production operations is perhaps largely dependent upon the quality and consistency of the semen product shipped to the breeding farms (Schulze et al., 2019). There are new tests that can be included into semen analysis that can provide information on a variety of sperm quality parameters. In the future, these measures may be useful for selecting high fertility boars, identifying fertility problems, or helping to adjust sperm numbers in the AI dose.

The use of alternative other forms of boar sperm, such as frozen, have not advanced as hoped, despite improvements in the production of doses, and the fertility with use of frozen sperm. Most of the improvements have occurred as a result of quality control in each of the steps for processing time, temperature regulation, and cooling and freezing formulas, and boar selection. However, despite the improvements in frozen sperm technology, when compared to liquid boar sperm, the reduced farrowing rates and litter sizes, the loss of semen doses produced per ejaculate, the time needed for dose preparation, and increased costs, remain as the major obstacles for advancing this technology. Nevertheless, there are circumstances with international gene transfer and restrictions to biological sample entering disease outbreak zones, where frozen semen would be required and most practical. Another form of boar sperm, sex-sorted, is of continued interest and value, but its use is still generally restricted to companies with the technology and use in research settings. While the ability to sort X and Y sperm is quite effective, the processing time is not fast enough to create doses with very high numbers of sperm needed for fertility success with swine AI. The key for this technology appears to be most practical if the numbers of sperm need can be reduced from billions to millions. To date, there have been fertility successes when using very low numbers of sperm with swine AI, but there is evidence that litter size declines as sperm numbers are reduced. To date, uterine or deep uterine inseminations with sex-semen in liquid or frozen for has met fertility expectations when using very low numbers in the AI dose. However, surgical insemination of sorted sperm into the oviduct using very low numbers has been effective for achieving good fertility, but its application is still limited by the time, cost, and labor required per animal inseminated.

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Reproductive Diagnostics

Diagnosing the reproductive status in female pigs at any stage of production would be a valuable for managing and troubleshooting breeding herd performance. Unfortunately, the methods are currently limited in most commercial operations to observation for estrus and farrowing events, and the use of ultrasound for pregnancy diagnosis at 4 weeks after breeding. Slaughter checks are also employed, especially when determining the cause for failed detection of estrus or reasons for pregnancy failures. But collectively, these tools provide little help when attempting to manage breeding herd failures, and for using precision in reproductive management. Use of real-time ultrasound to evaluate ovarian status and even uterine size has been an important tool for use in research for evaluating individual animals and has been employed as a veterinary diagnostic tool in some commercial farms around the world (Waberski et al., 2000; Martinat-Botté et al., 2003; Kauffold et al., 2004; Kauffold and Althouse, 2007). But the technology does require training, experience, and good quality equipment. The availability of new ultrasound units with higher quality electronics, that are smaller, portable and less expensive, will likely increase in the future. Perhaps their use for aiding in herd management and diagnostics will also then become more frequent in the future. There is the potential for use of other diagnostic tools, such as vaginal electrical resistance, or other physiological measures, that can be assessed for the reproductive tract and which can be associated with the reproductive steroids produced by the ovaries. Sampling for hormones in the blood, saliva, urine, or skin, whether intermittently or continuously could also provide important information on the reproductive activity of the ovaries. Collection and processing of samples, if required, would need to be easy and practical for routine use on farm. The assays to measure hormones would need to be quick, accurate, and inexpensive to allow for more routine use in management decisions or troubleshooting fertility. Some labs however, would also have access to more sophisticated assay systems that employ more rapid, and accurate assays for a wide range of hormones. Laboratory equipment employing chromatography, mass spectrometry and even nanotechnologies could dramatically advance diagnostics and even advance research in reproductive fertility. Even ELISA assays have advanced to the point that many can provide qualitative measures for rapid positive and negative results for hormones based on the use of dip-stick test strips. But in the future, implementation of any diagnostic technology will require a holistic view on the practical ability to obtaining the measure, and what decision can reliably be made to impact the efficiency for how the breeding herd operates.

Conclusions

Based on the need to meet animal welfare requirements, group housing of pigs in the breeding herd is likely to continue to increase around the world. Because of this, new estrus stimulation and detection systems will be important to test and evaluate. This will be an exciting area to watch evolve in the next years. There may be opportunities in these systems to improve the quality of stimulation and identification of fertility with the use of electronic technologies. These same technologies are also valued for their ability to monitor behaviors and physiology related to animal health, well-being, feed intake and body measures. In each case, these would also support optimal fertility. It is difficult to see major changes in AI technology, except for the continuing trends toward lower number of sperm per AI, a smaller AI volume, intrauterine AI, and use of semen without antibiotics. But major changes are always possible, and this could occur with advancements in diagnostics. If practical methods could provide real-time information of reproductive status such as estrus or ovulation, hormonal synchronization for groups of sows could occur allow by selection of eligible females for a single, timed AI. This same diagnostic approach might also allow for precision AI timing when using very low numbers of frozen or sex-sorted sperm. There are numerous possibilities for diagnostic approaches that could work, and many of these are being used and tested for application to human health. But those technologies that could be implemented for use in herd diagnostics, and that could easily and accurately identify fertility related events, in real-time, would be transformative for precision management of the breeding herd.

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Using computer vision to measure startle and freeze behaviour as indicators of pig emotion and welfare

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Introduction

Because concerns about animal welfare are, for many, based on an assumption that non-human animals can experience negative emotional states and therefore suffer (Duncan, 1996; Mendl, 2001; Dawkins, 2015), there is a particular need to develop validated indicators of animal affect (emotion). To this end, a variety of behavioural measures have been developed (e.g. human approach tests, novelty tests, cognitive bias, Qualitative Behavioural Assessment (Harding et al., 2004; Knierim and Winckler, 2009; Mendl et al., 2009; Battini et al., 2016; Phythian et al., 2016; Czycholl et al., 2017; Lagisz et al., 2020; Neville et al., 2020)). Some have been systematically validated against other indicators or by using manipulations of affective state grounded in a clearly-argued rationale, others less so (Richmond et al., 2017). In many cases, a major barrier to uptake under field conditions is the time required to collect and interpret the relevant data (de Vries et al., 2013; de Jong et al., 2016; Buijs et al., 2017). Consequently, there is still a need for new validated and reliable indicators of animal affect and welfare that are easy to implement under field conditions.

One potentially promising measure is the 'Defence Cascade' response shown to sudden, unexpected stimuli (Lang and Bradley, 2010; Kozlowska et al., 2015). This is a suite of adaptive responses evolved to ensure appropriate detection, evaluation and response to alerting stimuli. It involves initial detection and immediate response (*startle*) to a stimulus such as an unexpected noise (Lang and Bradley, 2010) followed by monitoring and evaluation of the stimulus accompanied by *freezing* / immobility (Lang and Bradley, 2010; Roelofs, 2017). Critically, startle and freeze are modulated by affective state in humans and rodents, making them potentially valuable indicators of these states.

In humans and rodents, negative states appear to potentiate startle magnitude. For example, humans show larger startle responses when viewing or anticipating unpleasant pictures (Vrana, 1994; Bradley et al., 2001; Grillon and Baas, 2003; Hurlemann et al., 2015; Nelson and Hajcak, 2017). In rodents, many studies demonstrate potentiated startle in subjects exposed to conditioned stimuli predicting an aversive event (Brown et al., 1951; Davis and Astrachan, 1978; Davis et al., 1993; Koch, 1999; Richardson et al., 1999; Grillon and Baas, 2003). Additionally, freezing behaviour in rodent aversive conditioning studies is frequently used as an indicator of a fear-like state (Phillips and Ledoux, 1994; Daldrup et al., 2015; Ross and Fletcher, 2018) and is potentiated by induced stress (Yee et al., 2012).

In contrast, startle magnitude is often attenuated in people exposed to pleasant pictures (Bradley et al., 2001; Hurlemann et al., 2015) and sounds (Bradley and Lang, 2000), and in rodents exposed to conditioned visual or olfactory stimuli associated with rewards such as food (Schmid et al., 1995; Koch et al., 1996; Schneider and Spanagel, 2008; Friemel et al., 2014). Likewise, freezing responses are attenuated by drugs and other treatments assumed to generate a less negative state (Hashimoto et al., 1996; Sartori et al., 2011; Osada et al., 2013; Modi et al., 2016; Ohyama et al., 2016).

Variations in startle and freeze behaviour thus offer potential as new indicators of affective valence and hence welfare. Preliminary studies of startle responses have been carried out in some farm animal species (e.g. pigs (Blackshaw et al., 1998; Statham et al., 2015; Carreras et al., 2017); cattle (Boissy et al., 2001); sheep (Desire et al., 2004)), and chickens (Ross et al., 2019). However, a major challenge to the use of these responses as indicators of affective state and welfare in the field is quick and accurate measurement. 'Gold standard' measures such as direct behavioural observations (e.g. detailed by-eye video analysis) are labour intensive and/or impractical under field conditions. One possible solution to this is to use computer vision techniques to implement real-time automated analysis of video-recorded startle and freeze behaviour. Successful development of this approach would allow cheap, objective and rapid measurement of these responses on farm, or in other contexts such as abattoirs, using just a video camera, a standardised eliciting stimulus, and the required software.

Here we explore this possibility in a commercially important farm animal – the pig. We chose the pig because it shows characteristic startle and freeze behaviour when unexpectedly disturbed, usually involving a whole-body startle and movement to a tense standing position followed by a period of immobility or freezing during which the animal appears

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to be monitoring or attempting to detect the source of the disturbance. These responses terminate when the pig flees or resumes ongoing behaviour.

We first provide a brief summary of a preliminary pharmacological 'proof of principle' study showing that pig startle and freeze behaviour may indeed be modified by affective state, as in humans and rodents, and therefore that successful development of an automated measure of these responses could open the way for using startle and freeze behaviour as an indicator of affective valence in pigs.

We then evaluate the validity of computer vision image analysis methods for measuring startle and freeze against 'ground truth' data provided by human behavioural observation which has been used successfully in previous studies of pig behaviour (Blackshaw et al., 1998; Statham et al., 2015; Carreras et al., 2017). We also collect force, kinematic, and depth-camera (Kinect) measures for comparison, as these are widely used to assess movement in other species. Finally, we evaluate whether contextual factors influence the responses.

Methods

Preliminary 'proof of principle' study: Pharmacological effects on pig startle and freeze responses
Twelve female pigs (mean 85kg) were housed in pairs. Each pair was exposed to two treatments: intra-muscular injection of both pigs with (i) an anxiolytic midazolam (M) 0.15mg/kg and (ii) saline control (C). Treatments were separated by 96 h and order was balanced across pairs. At 20, 35 and 50 mins following injection on each test day, the sound of a bursting balloon was administered to the pigs in their home pen and their responses video recorded. A simple measure of pixel movement was extracted from the videos to quantify the magnitude of the *startle* response made to the bursting balloon. *Freeze* durations were coded by-eye from the videos by a trained observer.

Main study: Validating the use of computer vision to quantify pig startle and freeze responses
Full details of Methods are available in (Statham et al., 2020). Here, we provide a summary of key points. The work was conducted under a UK Home Office Project Licence (PPL 30/2867) following review by the Bristol University Animal Welfare and Ethics Review Body.

Animals and housing. Twelve pigs (Large White x Landrace) were sourced from a commercial farm at approximately seven weeks of age. They were housed in two straw-bedded rooms (4.6m x 4.6m), naturally lit and supplemented with artificial light between 0700 and 1900, with a target temperature of 20˚C, and each holding three males and three females. Pigs were fed to appetite twice daily and water was provided ad libitum. They were weighed once a week.

Test room and equipment. The Test room contained a force-measuring pen and consisting of a 1.31 x 1.33m load platform fitted with four load cells surrounded by 1m high walls, three of which were made of 2" wire mesh whilst the fourth had a clear polycarbonate guillotine door through which pigs could enter. Fast capture video cameras (3 monochrome Point Grey Dragonfly Express cameras running at 200Hz) were positioned on two sides of the force-measuring pen and overhead, with the overhead position normalised by considering the location of the four corners of the pen. A standard video camera was set up for filming from one side of the pen. A Microsoft Kinect v1 camera running at 30Hz and collecting RGB and depth data was also positioned above the pen. During weeks 4 and 12 of the study, Kinematic data were collected using four infrared Qualisys cameras (ProReflex MCU240, Qualisys AB, Goteborg, Sweden) running at 200Hz and positioned at the four corners of the pen.

Testing. During weeks 1-2, the pigs were gradually habituated to human contact, moving to the Test Room and spending time on the load platform. Pigs were tested during two phases, initially when relatively young and light (weeks 3-5 of the study; 9-11 weeks of age; 20-40kg) and subsequently when approaching slaughter weight (weeks 11-13 of the study; 17-19 weeks of age; 50-80kg). In each phase, each pig received three Standard test sessions and one Kinematic test session. One pig had to be excluded from two sessions due to illness, thus giving 94 sessions in total. During each Standard test session each pig was taken from its home and entered the force-measuring pen. It was then given c.2.5 min to settle before the first test. A startling stimulus (e.g. bursting balloon) was then presented and the pig's response was recorded for 30s using the load platform, Kinect, fast-capture, and standard video cameras. Sessions comprised a maximum of 5 tests (stimulus presentations), each separated by c.5 min, after which the pig returned to its home pen. During Kinematic test sessions pigs were taken from their home pen to a small room where 10mm passive reflective spherical markers were attached to their backs using double sided sticky tape to allow motion-tracking. Once markers were securely in place the pig was taken to the Test room and the test procedure continued as for Standard tests, with kinematic data being recorded for 15s following each startling stimulus. In total, 285 tests nested within 94 test sessions (each comprising up to 5 stimulus presentations) were carried out across both phases.

Technical issues with some tests mean the final dataset for analysis was 280 tests.

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Data collection. Direct recordings from the standard video camera were coded by-eye using the Observer program (Noldus, 2011). An *Observer Startle Magnitude* score of 0-4 (least to most intense) was generated from the coded behaviour according to a rating scale. A total *Observer Freeze Duration* (s) was also calculated. Any freezes that lasted less than a second were reviewed and those less than 0.4s were re-classified as 'no freeze' because they did not show the tension / immobility required by our behavioural definition.

All Image Analysis (IA) measures were derived from the overhead fast capture camera (200Hz frame rate) which gave the best unobstructed view. Accelerations of highly textured image regions were estimated using sparse feature tracking. For freeze durations, both speed and acceleration were extracted. Sparse feature tracking considers only the easiest regions to track and hence improves accuracy compared to dense feature tracking (Shi and Tomasi, 1994). Birchfield's implementation of the Kanade Lucas Tomasi tracker was utilised (KLT; https://cecas.clemson.edu/~stb/klt/), and tracked points are referred to as KLT points. The magnitude of the initial response to the startling stimulus (*KLT Acceleration Startle Magnitude*; acceleration being closely related to force) was defined as the maximum acceleration of 50 KLT points (pixels/frame^2) in a temporal window 0.7 s after the startle stimulus. *KLT Speed Freeze Duration* was defined as the total time the speed of the 50 fastest KLT points (pixels/frame) was below an empirically determined threshold for a continuous period of at least 0.4 seconds. Thresholds were calculated using 10-fold cross validation with 90% training and 10% testing data repeated 10 times for all data.

Signals from the four transducers on the load platform were summed to give a measure of total instantaneous vertical force and normalised by the mass of each pig. *Load Platform Startle Magnitudes* were calculated as the peak absolute acceleration during a 0.7s window after the startle stimulus. *Load Platform Freeze Durations* were calculated using load platform acceleration measures and thresholding in the same way as for Image Analysis (IA) data.

Depth maps were extracted from Kinect data. For each depth map sequence, a time series recording the vertical displacement of the pigs' centroids for the total duration of each test was constructed. Vertical centroid speed was the magnitude of the first differential with respect to time in these series. Acceleration magnitude was the second differential. Startle magnitudes and freeze durations were extracted using both of these measures. *Kinect Speed* and *Acceleration Startle Magnitudes* were calculated as, respectively, the peak absolute values of the vertical centre of mass velocity (mm/frame), and acceleration (mm/frame^2) during a 0.7s window following the startle. The method used to calculate *Kinect Speed* and *Acceleration Freeze Durations* was the same as that used for the IA data.

Kinematic data were recorded for 15s following the startle stimulus and sub-sampled at 30Hz. *Kinematic Velocity and Acceleration Startle Magnitudes* were, respectively, the peak absolute values of vertical velocity (mm/frame) and acceleration (mm/frame^2) during a 0.7s window after the startle stimulus. Freeze durations were not calculated due to the short 15s recording window which failed to capture longer duration freezes.

Statistical analysis. The extracted variables in italics above were used in statistical analyses to compare automated readouts with observer ground truth measures for both startle magnitude and freeze duration. Due to inequality of variances and a highly skewed distribution of Load Platform data which was resistant to transformation, non-parametric statistics were used. We compared ground truth *Observer Startle Magnitude* scores to other measures of startle magnitude using Spearman Rank correlations. Given the hierarchical nature of the data, data points were not independent rendering derived p-values inaccurate. We therefore constructed multilevel regression models in MLwiN (Charlton et al., 2019) with Test (n=280) nested within Session (n=8) and Session nested within Pig (n=12). The response variable was *Observer Startle Magnitude* score (0-4), thus requiring use of an ordinal response multinomial model, with a reference category of 4 (Flee). In this model, negative estimated coefficients of predictor variables would therefore indicate a positive relationship with *Observer Startle Magnitude*. We carried out univariate analyses by adding each of the other measures of startle magnitude individually into the model as predictors of our response variable and used Wald tests to examine the significance of the term in the model and thus generate approximate p-values. The predictors were *KLT, Kinect, Kinematic* and *Load Platform Acceleration*, and *Kinect* and *Kinematic Velocity* estimates of startle magnitude.

For freeze duration, due to the large number of zero values present in this dataset, Spearman Rank correlations were again used to compare the ground truth *Observer Freeze Duration* with other measures. A multilevel model was constructed in MLwiN to derive p-values that accounted for the nested structure of the data, with *Observer Freeze Duration* as the response variable. Although the data were not amenable to being transformed to normality, we used a normal rather than binary model to allow comparison to the correlation results. In this model, a positive estimated coefficient of a predictor variable indicated a positive relationship with the response variable. The predictors were *Kinect, KLT* and *Load Platform Acceleration*, *Kinect Speed,* and *KLT Speed* estimates of freeze duration.

Multilevel models were used to investigate the possible effects of a range of factors (e.g. pig sex, weight, behaviour prior to test, orientation in apparatus, time of day, experience of test across time and within a day, startle stimulus, test

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session type) on observer measures of startle and freeze responses. As above, an ordinal response multinomial model was used for startle magnitude data with *Observer Startle Magnitude* (0-4) as the response variable. However for *Observer Freeze Duration* data, where the comparison to continuous measures required in the above analyses was now not essential, we converted the non-normal data into a binary freeze / no-freeze variable and used a binomial model with a logit link function. The hierarchical structure of the data was as described previously.

Results

Preliminary 'proof of principle' study: Pharmacological effects on pig startle and freeze responses
There was no effect of midazolam (M) treatment on the observed activity of the pigs relative to saline controls (C) *prior* to administration of the startling stimulus (e.g. standing / rooting: M (24/36 tests), C (25/36 tests); walking: M (3/36 tests), C (5/36 tests), chi-square on all measures=10.2, df=7, NS), or on computer image analysis measures of pixel movement at this time (M=3.46±0.54, C=4.67±0.67, $F_{1,4}$=0.799, NS), indicating no baseline differences in activity.

However, repeated-measures ANOVA with treatment as a between-subjects factor showed that M pigs exhibited a significantly smaller initial startle reaction, measured by computer image analysis of pixel movement during 1s following the sound stimulus (M=7.08±1.08, C=14.70±1.44, $F_{1,4}$=53.1, P=0.002; Figure 1a), and a significantly shorter freezing response (M=0.37±0.26, C=2.07±0.59, $F_{1,4}$=9.5, P=0.037; Figure 1b) and time to resume ongoing activity (M=0.90±0.37, C=4.86±0.89, $F_{1,4}$=10.96, P=0.03; Figure 1c), both measured in seconds.
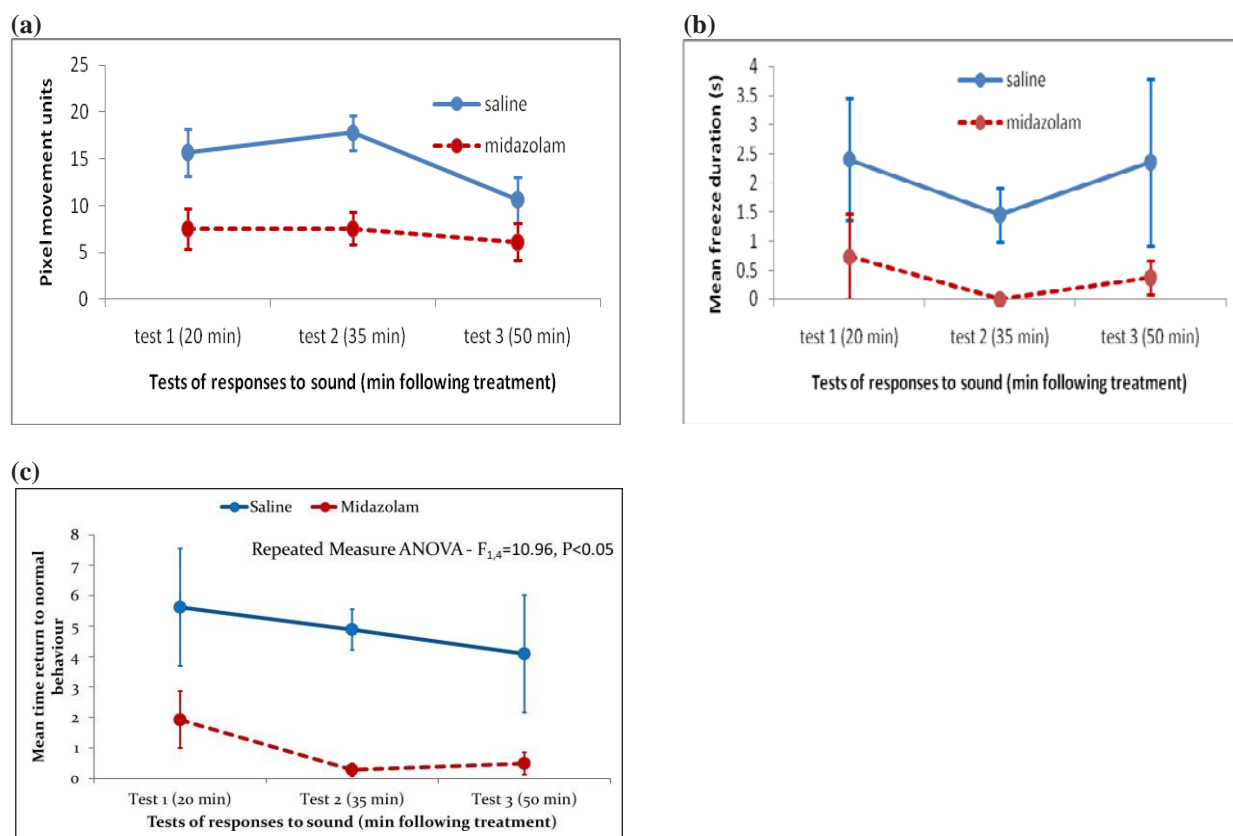
(a)

(b)

(c)

Figure 1. Behaviour of pigs following injection at time 0 mins with either saline (N=12, solid lines) or the anxiolyticmidazolam (N=12, dashed lines), when the starling stimulus was applied at times 20, 35 and 50 mins: (a) computer image readout of pixel movement during 1s following the three startling stimuli (arbitrary units); (b) by-eye coded measure of freeze duration (s); (c) by-eye coded measure of time taken to return to normal behaviour (s).

Main study: Validating the use of computer vision to quantify pig startle and freeze responses

Comparisons of automated readouts with observer ground truth measures – startle magnitude. The ground truth Observer Startle Magnitude measure was significantly positively correlated with all automated measures of startle magnitude (Table 1). The strongest correlation was with the Kinematic measures, although these were only available for a subset of the data. Multilevel models showed that the Load Platform and then KLT Acceleration measures were the strongest predictors of the Observer Startle Magnitude scores. (Table 1). KLT Acceleration data

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were strongly positively correlated not just with Observer Startle Magnitude, but also with all the automated measures, which are widely used to measure movement (Load Platform, r_S=0.824: n=280, P<0.001; Kinematic Velocity: r_S=0.821, n=70, P<0.001; Kinematic Acceleration: r_S=0.816, n=70, P<0.001; Kinect Speed: r_S=0.772, n=280, P<0.001; Kinect Acceleration: r_S=0.693, n=280, P<0.001; Figure 1).

Table 1. Relationships between the Observer Startle Magnitude score and the Kinect, Kinematic, Load Platform and KLT estimates of startle magnitude.

Measure	n	Corr. Coef. (p-value)	Coefficient estimate (with SE)	Wald X^2 (p-value)
Kinect Acceleration	280	0.529 (<0.001)	-0.395 (0.047)	70.126 (<0.001)
Kinect Speed	280	0.606 (<0.001)	-0.243 (0.028)	75.447 (<0.001)
Kinematic Acceleration	70	0.743 (<0.001)	-1.013 (0.166)	37.103 (<0.001)
Kinematic Velocity	70	0.729 (<0.001)	-0.519 (0.088)	34.557 (<0.001)
Load Platform	280	0.706 (<0.001)	-5.565 (0.524)	112.978 (<0.001)
KLT Acceleration	280	0.654 (<0.001)	-0.847 (0.088)	92.855 (<0.001)

Correlation Coefficients and associated p-values were calculated using a Spearman Rank Correlation. The Coefficient Estimate,related Standard Error, Wald statistic and associated p-value were from the ordered multinomial multilevel models.
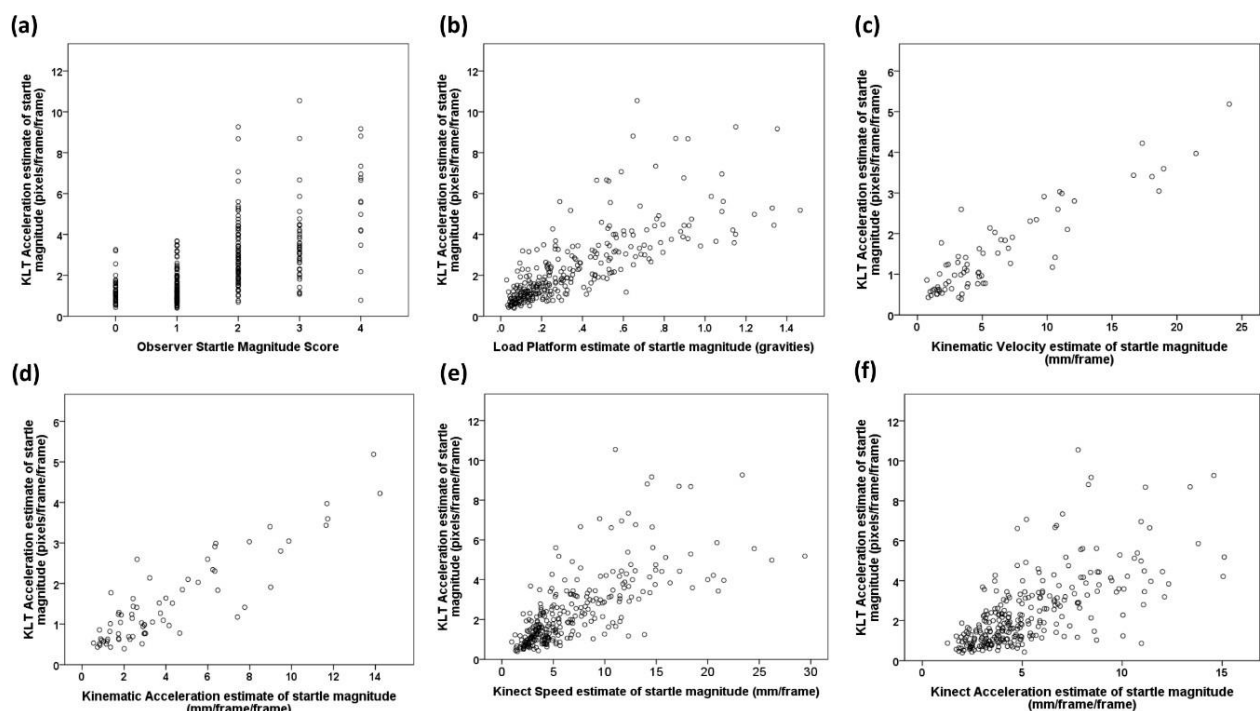

Figure 1. Scattergrams of the relationship between KLT Acceleration estimates of startle magnitudes (pixels/frame^2) and those provided by (a) Observer Startle Magnitudes scores; and (b) Load Platform (gravities); (c) Kinematic Velocity (mm/frame); (d) Kinematic Acceleration (mm/frame^2); (e) Kinect Speed (mm/frame); (f) Kinect Acceleration estimates (mm/frame^2).

Comparisons of automated readouts with observer ground truth measures – freeze duration. The ground truth Observer Freeze Duration measure was significantly and positively correlated with the Kinect, Load Platform and KLT measures. Multilevel models showed that the Kinect Speed and then KLT Speed measures were the strongest predictors of the Observer Freeze Duration scores. (Table 2). KLT Speed estimates of freeze duration were strongly positively correlated with those derived from the other automated measures, when both measures detected a freeze (Kinect Speed: r_S=0.938, n=96, P<0.001; Kinect Acceleration: r_S=0.879, n=98, P<0.001; Load Platform: r_S=0.894, n=80, P<0.001 Figure 2). We did not calculate corresponding KLT Acceleration correlations due to the low sensitivity of this measure.

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Table 2. Relationships between the Observer Freeze Duration measure and the Kinect, Load Platform and KLT estimates of freeze duration.

Measure	n	Corr. Coef. (p-value)	Coefficient estimate (with SE)	Wald X^2 (p-value)
Kinect Acceleration	282	0.837 (<0.001)	0.876 (0.030)	381.299 (<0.001)
Kinect Speed	282	0.833 (<0.001)	0.867 (0.027)	422.209 (<0.001)
Load Platform	283	0.784 (<0.001)	1.030 (0.0.34)	397.375 (<0.001)
KLT Acceleration	283	0.771 (<0.001)	0.908 (0.034)	351.285 (<0.001)
KLT Speed	283	0.821 (<0.001)	0.848 (0.027)	412.752 (<0.001)

Correlation Coefficients and associated p-values were calculated using a Spearman Rank Correlation. The Coefficient Estimate, related Standard Error, Wald statistic and associated p-value were from the multilevel models.
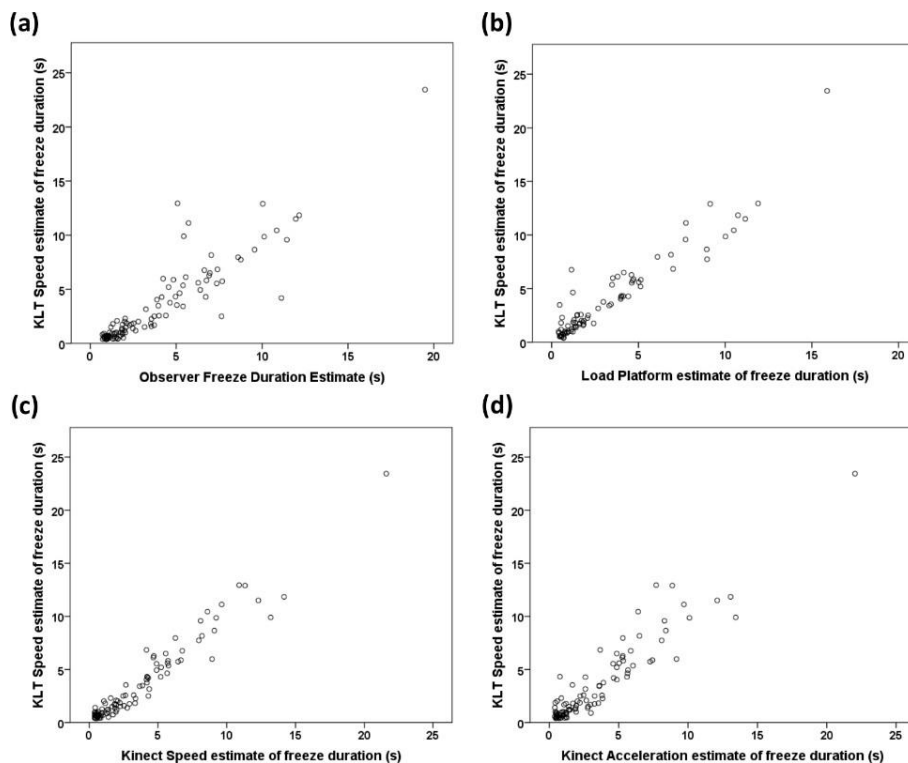

Figure 2. Scattergrams of the relationship between KLT Speed estimates of freeze durations and those provided by (a) Observer; (b) Load Platform; (c) Kinect Speed; (d) Kinect Acceleration estimates.

Overall, computer vision KLT Acceleration estimates of startle magnitude, and KLT Speed estimates of freeze duration, correlated well with the corresponding Observer ground truth measures and with Load Platform, Kinematic and Kinect measures of pig movement.

Effects of other factors on the observer measure of startle magnitude. Session had a significant effect on Observer Startle Magnitude scores (Wald X^2= 21.678; P<0.005) due to stronger startle magnitudes occurring in Session 4, when the startling stimulus was altered to overcome habituation to the original bursting balloon. The use of new stimuli (e.g. a moving bin-liner) resulted in increased startle magnitudes (Wald X^2; 22.54; P<0.001). When compared to pigs that were rated as being calm prior to presentation of the first startling stimulus (relaxed-tense score of 1), those that received a score of 2 or 3 displayed a stronger startle reaction, but this effect was not seen for those animals with scores of 4 and 5 (Wald X^2= 19.219; P<0.001). Larger startle reactions were seen when the pig was orientated away rather than towards the startling stimulus (Wald X^2= 14.827; P<0.001).

Effects of other factors on the observer measure of whether a freeze response occurred. The likelihood of a freeze response decreased from Session 1 to 2 and increased significantly in Session 4 when the startling stimuli started to be varied (Wald X^2= 26.013; P<0.001). There was also an increased likelihood of a freeze response when the pig was facing away rather than towards the stimulus at the point of testing (Wald X^2= 8.735; P<0.05), and for pigs with a 'relaxed-tense' score of 2 or 3 compared to a score of 1 prior to the first startling stimulus (Wald X^2= 16.824; P<0.005). However, pigs with a score of 4 or 5 did not show this increased likelihood of freezing.

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Discussion

Our preliminary 'proof-of-concept' study indicated that whilst application of the midazolam anxiolytic did not appear to reduce the general activity levels of pigs compared to when they received saline, it significantly affected their responses to startling stimuli. Pigs receiving midazolam showed a smaller *startle* response to the bursting balloon, a shorter *freeze* response and were quicker to return to normal activity. These findings clearly support the hypothesis, based on findings from human and rodent studies, that pigs in a presumed less anxious (more positive) state show attenuated startle and freeze components of the defence cascade response. Moreover, because these pigs did not differ in baseline activity, froze for shorter periods of time, and returned to normal activity more quickly than saline controls, there was no evidence that the anxioloytic was exerting a general sedative effect. Startle and freeze responses thus show promise as new indicators of affective state and welfare in pigs.

Given this finding, we investigated the validity of computer vision image analysis (IA) as a measure of startle magnitude and freeze duration responses in pigs, because of its potential as a practical tool for assessing these responses under field conditions. We used behavioural observation of video as our ground truth measure of startle and freeze responses (Blackshaw et al., 1998; Statham et al., 2015; Carreras et al., 2017) and also collected load platform, kinematic, and Kinect depth-camera measures because these have been used to assess movement in other species.

Our KLT Acceleration image analysis estimate of startle magnitude, and estimates from all the automated measurements we made, were significantly positively correlated with the 'gold standard' observer behavioural observation data. The image analysis estimate of startle magnitude was also strongly positively correlated with the other automatically recorded data from load platform, kinematic and depth-camera sources.

Our KLT Speed and Acceleration image analysis estimates of freeze duration were strongly positively correlated with the observer measure, as were Kinect Acceleration and Speed, and Load Platform data. However, it was clear that there were false negative results, for example when the observer recorded short freezes (<2s) in which the body appeared tensed but isolated parts (e.g. ears) were moving. This short duration and residual movement may have accounted for the failure of the automated measures to detect a freeze response. When just looking at the true positive data (when both the observer and automated measures detected a freeze), automated measures of freeze duration were strongly positively correlated with those made by the observer, with KLT Speed and Kinect Speed performing best. Furthermore, KLT Speed estimates of freeze duration were also strongly positively correlated with the other automated measures.

In addition to analysing the relationship between different measures of startle and freeze behaviour, we also investigated factors that may influence expression of these behaviours in pigs tested under laboratory conditions. Some habituation to the original startling stimulus (bursting balloon) meant that we needed to vary the nature of the stimulus within test sessions, starting in session 4, and startle response magnitude increased after this session. Similarly, the likelihood of a freeze response initially decreased from session 1 to session 2 when the bursting balloon was used repeatedly, but increased in session 4 when stimuli started to be varied within sessions. The type of stimulus used also affected DC responses with bin-liner and umbrella stimuli, both involving rapid movement, being particularly effective at generating greater startle magnitudes than the balloon. Use of stimuli with a pronounced visual component may thus be more potent inducers of DC responses in pigs compared to purely auditory stimuli.

Larger startle magnitudes and an increased probability of freezing were observed when pigs were orientated away from the stimulus. One potential explanation is that startling stimuli presented in this context induced a greater surprise reaction with increased post-startle processing required to resolve the source of the event (Lang and Bradley, 2010). The effect on startle magnitude may also have resulted from the fact that observer scores of 'jump with movement' (e.g. re-orientating towards the stimulus) were ranked as reflecting a larger startle response than 'jump on the spot' (more likely if the pig was already facing the stimulus).

A subjective rating of how 'relaxed or tense' the pigs were before the first stimulus was delivered during a test also influenced DC responses. For both startle magnitude and freeze occurrence an increase in reaction was seen for pigs scored as 2 or 3 when compared to the calmest pigs (scored as 1). However, this difference was not observed in pigs scored as 4 or 5 (least calm). In fact, the probability of a freeze reaction was decreased in pigs with a score of 4 compared to those with a score of 1. Although this seems counterintuitive, we observed informally that the least calm pigs appeared to pay least attention to their surroundings and were often attempting to leave the test pen. Consequently, delivery of the startling stimulus did not intrude into their already active behaviour. This re-emphasises the need for pigs to be settled and calm at the point of testing, for example in a home pen.

It is interesting to note that our models found corresponding effects of moderating factors on both startle magnitude and probability of freeze occurrence. Both increased when startling stimuli were altered, when the pig was facing away from them, and in pigs who were slightly or moderately tense prior to their presentation, although not for animals

rated as tense or very tense who appeared to be focused on leaving the test pen. This coherence of effects indicates that startle magnitude and freeze probability may reflect a similar underlying construct, for example affective valence (Koch, 1999; Richardson et al., 1999; Winslow et al., 2002; Grillon and Baas, 2003).

Conclusions

A preliminary 'proof-of-concept' study indicated that pig startle and freeze behaviour appears to be modulated by affective state in a similar way to that observed in humans and rodent. To progress work in this area, validated automated computer vision measures of pig startle and freeze behaviour will be valuable from both an experimental perspective and for quantifying this behaviour under field conditions. To this end, we found that computer vision image analysis measures were comparable with force, kinematic and depth measurements at estimating ground truth observer measures of both startle magnitude and freeze duration / occurrence. These findings demonstrate that computerised image analysis of video recordings can be used to detect and quantify startle and freeze responses in individual pigs, and hence has potential as a practical automated measure of these behaviours under field conditions. We also identified factors including startling stimulus characteristics, orientation relative to the stimulus, and 'relaxed-tense' behaviour that can moderate startle and freeze responses and hence should be borne in mind during further investigation of this behaviour. The similarity of these moderating effects across both startle and freeze behaviours indicates that they may represent similar underlying constructs such as affective valence. If variation in startle and freeze behaviour does indeed reflect emotional state in pigs, as it appears to in other species, computer vision image analysis may provide a novel method for assessing pig welfare in the field. Further research on the link between affective state and pig startle and freeze behaviour, the capacity of image analysis to measure these behaviours in groups of pigs, and the utility of the approach under field conditions is needed to realise this potential (Blokhuis et al., 2010; Statham et al., 2015; Statham et al., 2018).

Acknowledgements

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Digitalization, pig data and the internet of the swine things.
The future is now for pig Vets

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The world has changed in the last two years to the extent that no one from the *last two* generation onwards have experienced before. This change has been mostly related to the COVID19 pandemic but also have been determined by other concurrent factors, including the rising costs of energy (fuel, electric power) and the alteration of global supply chains. The livestock sector adds some specific factors that interact with the prior mentioned and all among them. This includes the spread of several catastrophic animal diseases like African Swine Fever in Asia, Europe and Central America, the latest outbreaks of Avian influenza, the raising of antimicrobial resistance and the stronger regulations in the use of antibiotics in the industry, the growing environmental regulations affecting not only emissions into the soil, water and air, in particular, those related to global warming because of greenhouse gasses emissions (GHG) in terms of CO2 eq and transboundary pollution in terms of N-ammonia related emissions and, also, the already existing and upcoming animal welfare regulations like the California Proposition 12, Animal Confinement Initiative or the open discussion about banning cages in the EU. Within this already complex enough scenario, we must add the pressure of the social network, promoted by animal activists' parties against the industrial livestock sector, being pig production one of their main targets, using skilled and biased pictures and videos of very specific and unusual situations as a fair theoretical representation of the activity of the sector, aiming to generate negative emotions in the consumers.

On the other side, it is pretty clear that the pig sector is one of the necessary contributors to feed the world (37 % of the meat consumed), producing high-quality protein and some other nutrients associated by only generating 1-3 % of the CO2 eq, depending on the source consulted (FAO, 2018, EPA 2019) and its activity it is supported by millions of direct and indirect jobs worldwide (Figure 1).
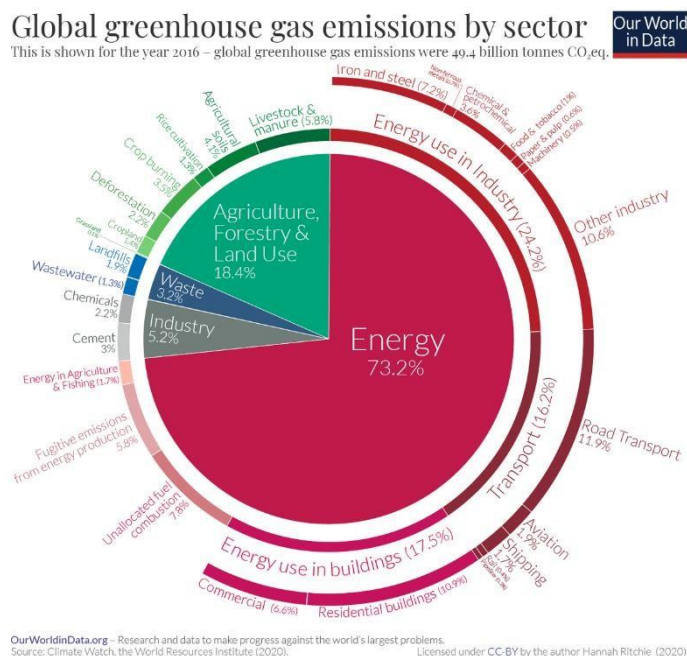

Figure 1. Global greenhouses emissions by sector

So how can we match the two needs in this challenging and demanding scenario? A recent publication from the consultancy firm Deloitte in 2019 gives us some clues to face this problem. Since the publication was released just before the pandemic, it is not considering some of the current facts cited above, but most of the conclusions and recommendations are perfectly valid or even more than then. The report describes the over time expected demand for meat (total, not only pork), that is mainly driven by continuous population growth and rising per capita incomes and how the sector must fulfil the future demand, ensuring quality in a sustainable manner, without exceeding global resources and avoiding irreparable environmental damages.

The report is looking to 2050 situation describing the likely situation then with 9.7 billion people with an average income of 30,333 $ US and global consumption of 498 million tons of meat instead of the 334 described in 2015, with an increased per capita consumption of 13 % from 45 to 51 kg per head per year. This is expected to happen more in Africa (2.5 fold) and in Asia (+50 %). It is also well described the impact of livestock in land use, manure excretion, the lack of efficient logistics, water use and the GHG. Since the report was released a bit before the pandemic is not considering its impact and other factors but also includes the regular low prices of meat and dairy products, the volatility on the demand, the increased needs related to animal welfare and the information from the customers about the food that they consume is produced and the reduction in the use of antimicrobials. Interestingly, the report states that we will have enough land or water to meet this demand, even double it, but the main constrain will be GHG emissions. The message is quite straight, affirming that if global emission growth continues the same way, GHG emissions will reach 139 Gt in 2100, and the earth temperature will be 4.5 ℃ above pre-industrial levels. The meat sector plays a crucial role in achieving these objectives, as it accounts for almost 10 % of the emissions. Under the business-as-usual model (BAU), meat emissions would increase from 5.2 Gt per year today to almost 8 Gt in 2050. To keep global warming below 2º C, meat emissions have to be decreased to 3.2 Gt by 2050. Under BA and keeping the emissions under this threshold, only 41 % of the meat required can be produced (Figure 2).
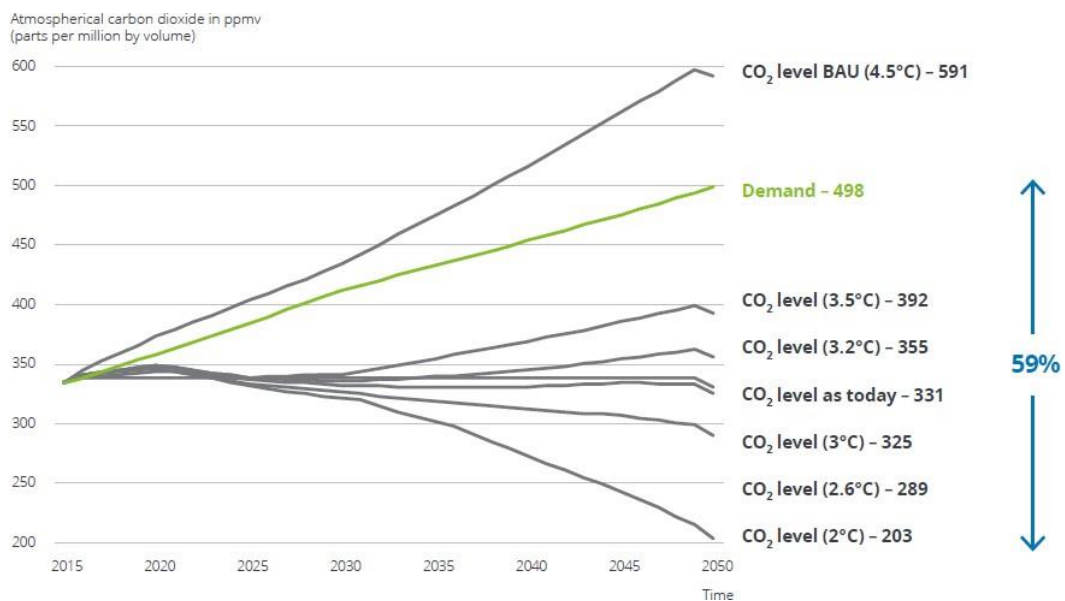

Figure 2. CO2 levels in the atmosphere, temperature associated and percentage of meat demand to be achieved (Deloitte, 2019)

To produce the rest 59 % and keep the emissions limited, a number of approaches must be implemented, having every of them a measured impact in the CO2 emissions and it is the successive implementation of these measure what will allow to meet the objectives (Figure 3).
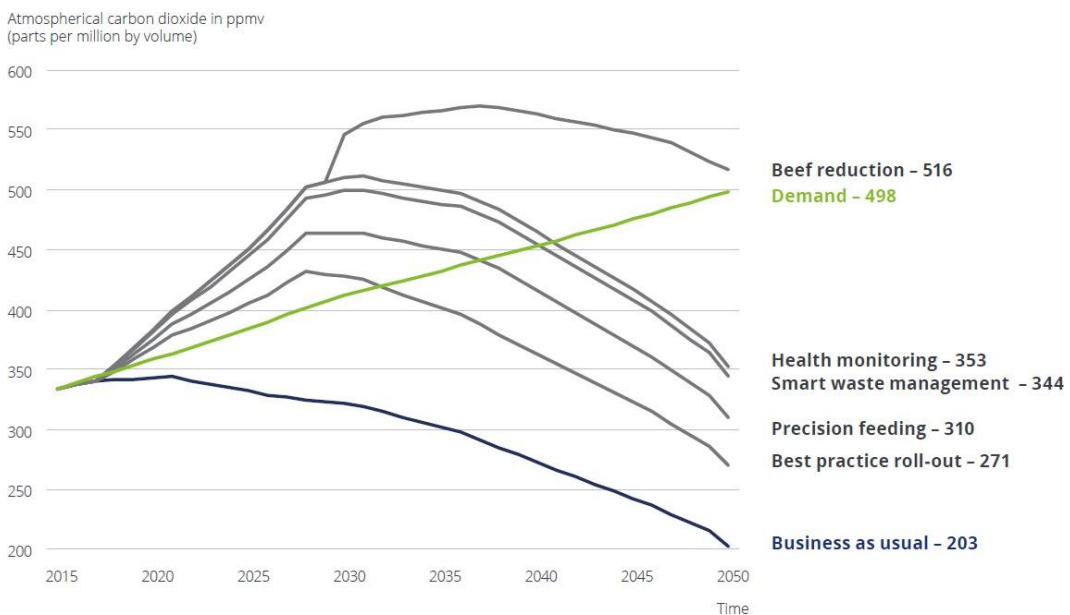

Figure 3. CO2 levels in the atmosphere associated with abatement options (Deloitte, 2019)

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But how to achieve these objectives? The reports also suggest the ways and in general are closely related to the digital transformation, which is related to rethinking the productive process based on the use of digital tools (those that produces data, being either hardware-farm equipment or software), and it is affecting the whole organization

By combining best practices (25 % of improvement), precision feeding (13 %) and smart animal health and welfare a good share of these objectives can be achieved. And now the question is how to put it into practice? The report refers to several technological enhancements, including the use of information and communication technologies (ICT), Cyber-physical systems and the Internet of the things (IoT, but I would say better Internet of swine things, IoST), big data analytics, platforms, cloud computing and artificial intelligence. As a consequence of the digital business models, can meet not only the desired objectives in terms of GHG emissions but also improve the benefits before taxes (EBITDA) of the companies up to 30 % (Figure 4).
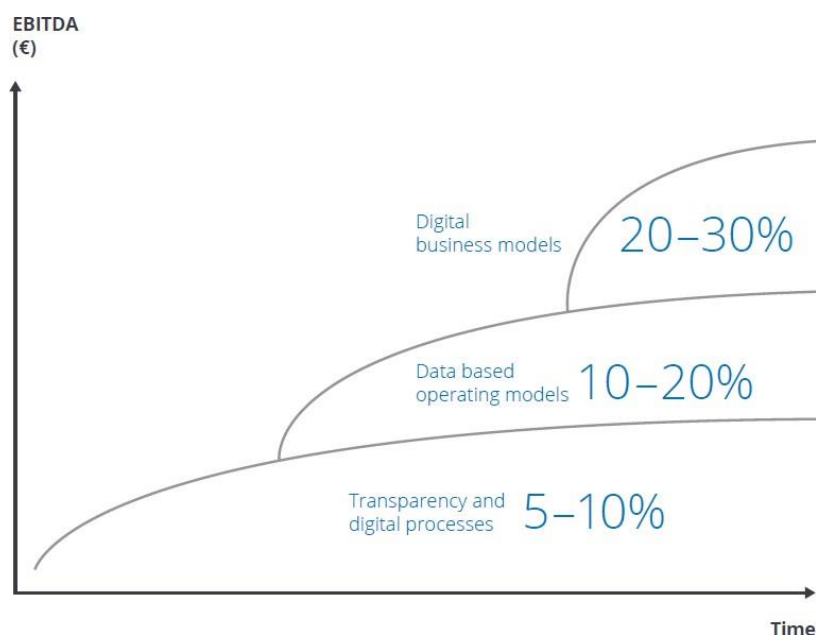

Figure 4. EBITDA associated to the implementation of digitalization steps (Deloitte, 2019)

But how to put this in practice in our swine sector?
During the last decades, the use of data by farmers has been limited. Most of the systems used were simple and mainly focused on the management of farm tasks, with limited or no capacity for analysis. Integration of data from different sources or farms was also difficult, and there was little applied knowledge on the value of data in strategic decision-making. Another weak point not solved so far is the lack of support services in the use of data promoting digital transformation and the implementation of systems of information management. The first steps and topics to address are presented in Figure 5.
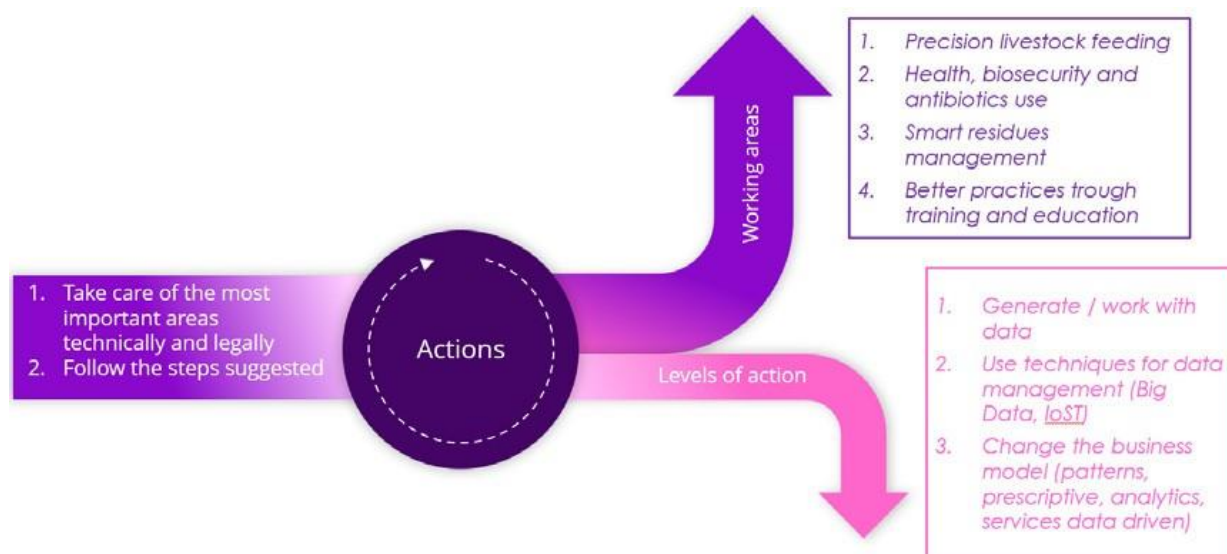

Figure 5. Working areas and levels of action derived from digitalization implementation to address sustainability requirements

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The use of data in many agricultural crops has exploded in recent years; however, its use in livestock health and production is still limited. In pigs, data collection has not changed for many years and analysis is still focused on the main reproductive key performance indicators (KPIs) such as farrowing rate, the number of repeat services, total born, born alive, stillborn, mummifies, weaning to first service interval and preweaning mortality. Other types of data, such as environmental or slaughterhouse data, data from feeding stations or other automatic sources, have not been used in practice except to create simple alerts like detection of temperatures out of range or sows that have not eaten. Among the reasons for this lack of progress is the low added value perceived by producers, the good margins that for years prevented the need for improvement based on production data analysis, the lack of professionals with solid education on-farm data management or the lack of tools to facilitate the process of extracting value, benchmarking and monitoring. On top of these issues, companies manufacturing farm equipment and software that generate data did not facilitate its extraction and use, rather the opposite to protect their equipment and systems.

The Five Steps in a Swine Management System

Data must be transformed into information to generate knowledge. It is not the same, although many times is confused. Therefore, there are many companies' rich in data but poor in information'. To avoid this, companies of any size must set their own information system to solidly and routinely support their decision-making process at any level. A swine management information system can be defined as *'A system made up of tools (software and devices) that together with a working protocol and procedures, including the roles of users, can generate the necessary information to diminish the risk and uncertainties in decision-making'*. Such a system always has five steps (Figure 6), independently of the size and characteristics of the company that uses it:

- **Step 1, Data collection**. Data is the raw material of the system. Must be of quantity and quality enough and can come from human inputs or sensor and robots. Until now, data is just numbers, but the sector is coming closer to the use of images (Farmsee®, Israel) and sounds (respiratory distress detected by Sound talks™, Sound talks, Leuven, Belgium).

- **Step 2, Data processing**. Includes several tasks like management of outliers, missing data and use of different formats from different sources. The objective should be the adequate set-up of database structures that allow proper use and interoperability of data (data sharing across systems).

- **Step 3, Reporting**. Deciding and producing the type of reports of interest for the farm or company at every level is not a minor task. From sow cards or working lists (i.e. sows to be mated or vaccinated) up to multivariate regression analysis to define the optimum value for a certain KPI (i.e. age at first mating considering several variables), every farm or company must decide the reports needed by every work level (farm staff, farm manager, veterinarian, technical manager, board of directors or chief executive officers), not forgetting that could be just technical, economical or mixed.

- **Step 4, Distribution of the information**. The objective of this step would be sending the right information to the right person at the right time and using the right channel. This is not properly done in many cases and is an overlooked reason for the underuse of data. Sometimes information arrives a bit late and is useless (i.e. hypo-productive sows to be culled if report arrives once mated) or is too complex for farm staff or too simple for veterinarians or managers. User preferences to receive it must be considered as well and can include from classical PDF files, text messages at the smartphone or web applications. Every user will be more comfortable and will make better use of the communication channel is the most adequate.

- **Step 5, Analytics and decision-making**. Information received must be read, understood and used by a person with the right education and with time enough to make a decision to be implemented. Until now, analytics were aimed to be mainly explanatory, but predictive analytics is becoming a key step in most industries due to the amount of quality data available using artificial intelligence techniques such as machine learning (an application that provides systems the ability to automatically learn and improve from experience without being explicitly programmed) or artificial neural networks (an information processing paradigm that is inspired by the way biological nervous systems, like the brain, process information. It is composed of a large number of highly interconnected processing elements (neurones) working in unison to solve specific problems).

Figure 6. The five steps of an information system

Every farm or company must follow these five steps to establish a robust information system that supports its production efficiency and required quality standards. The information generated and analyzed can be structured in five levels and, in an information system well designed, all of them can obtained from the same database. The 4 levels are the following:

1) Alerts. A KPI goes beyond or below a certain threshold generating an alert looking for immediate intervention. Normally are based on text messages that can be easily generated automatically. Regularly present in the industry. Examples are the mortality rate (pre-weaning, sows or nursery grow-finish), repeats %, abortions %, and the number of piglets born alive or weaned.

2) Monitoring. It is related to what is going on regarding a certain KPI. Normally based in time evolution generating charts, normally with no statistical treatment at all, being just graphical representation of the evolution. Typical examples are similar to alerts, including farrowing rate, prolificacy (total born, alive or still), feed per sow per year

3) Explanatory analytics. Data with a statistical analysis of any kind, from descriptive, comparisons, time-series or regression usually. This analysis is in general uncommon and not applied in a routine and, therefore, is unfrequent to be sure or reasonably sure over the KPIs analyzed, despite the importance of the actions derived from it.

4) Predictive analytics. When there is data enough of quality enough with a certain degree and complexity, and we want to treat them at the high velocity possible to make or anticipate funded decisions, there is the adequate background to run predictive analytics, including decision trees, regression or neural networks, existing several models for this (classification, clustering, forecast, outliers and time series) and the most common algorithms (machine learning and deep learning) can be related to one or more of the models (random forest, k-means, GLM for two values).

5) Prescriptive analytics. Not existing yet in our sector. Has been defined as predictive analytics + a working protocol, meaning something like 'if this is likely to happen, in this case you should do that'.
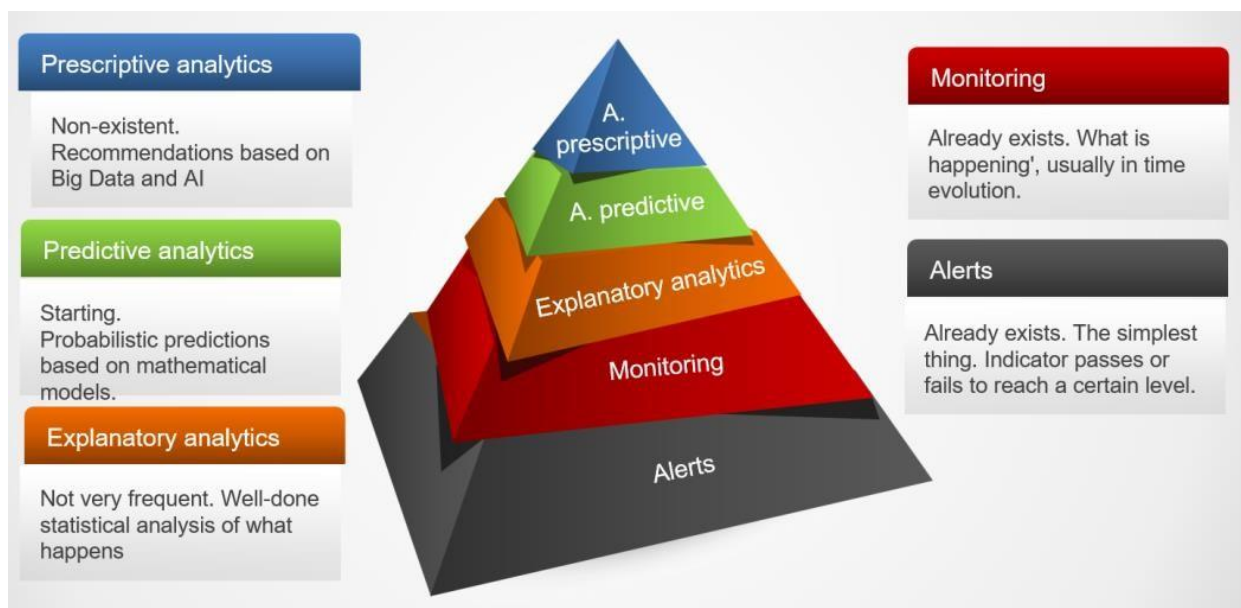
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Figure 7. The five levels of information generated from data

It is interesting how models coming from other sectors can be applied to swine health and production, including logistic regression to predict the performance of the reproductive sows (Wakatsuki et al., 2021) or the probability of a respiratory or enteric outbreak based on climate indicators (Rodríguez et al., 2021). These models can be applied to forecast the behavior of certain KPIs, including piglets born alive, stillborn, or sows' mortality, among others and are more valuable y with include risk factors or metadata y the database. Examples of high interest are the type of production (farrow to finish or multisite, gilts rearing or purchase or farm size), health status (PRRS positive), vaccination or medication program or type of sows housing (stalls, stanchions, ESF-tunnel, ESF-autocapture) clustering farms based on them. This will allow a faster, better and more reliable understanding of the impact of the factors studied.

The need for new technologies

In the last decade, the productive global framework has been changing. New technologies have been developed in all sectors and are finally reaching livestock farming. Moreover, producers are becoming aware that their competitiveness depends on using their data appropriately to support their decision-making, both for daily decisions as well as strategic ones to improve their competitiveness. The current productive demands are forcing producers to optimize all aspects of the productive chain. But which are the available data sources, and which will be soon? Among the existing ones, probably the most traditional and known is reproductive.

The modern sow from different breeding companies, is quite different from the animals we managed 1 or 2 decades ago. It is much more productive in terms of the number of piglets born and weaned, having therefore higher nutritional needs but also has a lower feed intake which comprises fetal development and milk production. Understanding the modern sow can be partly done by means of big data analytics, defining better the most relevant KPIs and which are the risk factors that explain them better to optimize their performance under commercial conditions. Based on this approach in the recent years has been better defined:
- Averages, trends and patterns in sows' mortality and which are the risk factors that influence more (Tani et al., 2017a)
- Sows' lameness prevalence and its impact on production (Iida et al., 2020)
- Preweaning mortality average and patterns change over time (Koketsu et al., 2021)
- Risk factors for severe repeat-breeders sows and their lifetime performance (Tani et al., 2017b)
- Abortion occurrence and factors associated (Iida et al., 2016)
- Weaning to estrus interval influence on lifetime performance (Yatabe et al., 2019)

And not only explanatory analytics but also prediction has been performed in some cases using machine learning algorithms, as logistic regression.
- Prediction of a lifetime performance of a sow based on her first parity (Iida et al., 2015)
- Prediction of fertility performance based on timing and temperature thresholds of heat stress effects.

Since it is in reproduction where more data of higher quality are generally available will be the first place where artificial intelligence models will arise that will not replace but will help vets to deliver a better consultancy. A recent example has been developed together with some of our customers to quantify and prioritize the areas of improvement based on the weight of every variable for weaned piglets per sow per year (WPSY) for every farm, subset or whole

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companies based on their own data and the productivity trees associated. With this algorithm, our team of vets and consultants can understand better which are the most important and prioritize actions to achieve the desired improvements in that particular farm or company, being a great combination of digital tools + vet mindset and knowledge to generate better business. Figure 8 shows the relationship among the variables helping to decide where to focus the work to improve the WPSY.
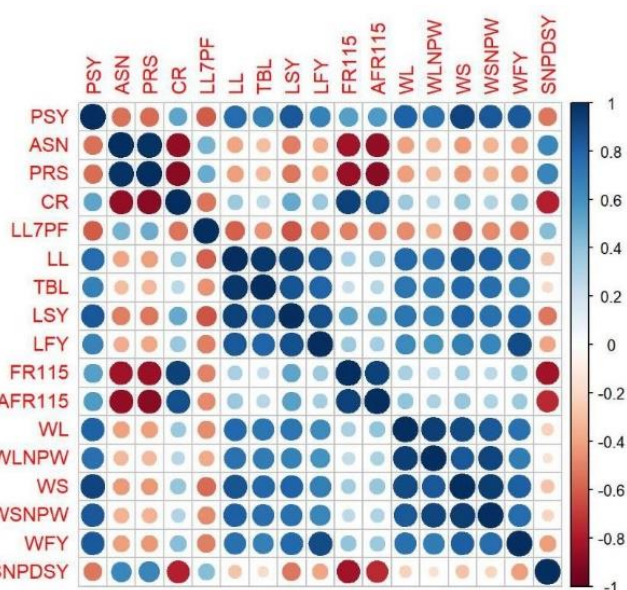

Figure 8. Correlation between variables for WPSY

How can veterinarians use these data? There are several ways to get benefits from them. The first one is using individual farms data to understand what is going on, which are the risks affecting the main KPI and how to facilitate production flow. The second one is merging farms based on certain interest factors (breed, production type, health status, among others) and monitor performance. The third one is to use these AI techniques on big data to predict performance and quantify risks, but this will only be possible with data scientists.

Feeding data

Feeding data is one the most important parts of precision livestock farming (PLF). In this regard, as described by Wathes et al. (2008) can be defined as the management of livestock production using the principles and technology of process engineering and is the principal means by which 'smart' sensors will be used in livestock farming. This PLFis also known as "Integrated Management Systems" and is based on automatic monitoring of livestock and related physical processes. This concept has come to address some of the shortcomings in data generation and processing and has converged with the global trend towards the digitalization of many products and services. The development of PLF concept has allowed, in a short period (last five years), to face a very different scenario when it comes to being able to take advantage of the information generated from data produced in the sector.

Feeding data has been traditionally managed by using averages of feed delivery both to sows, piglets and finishers and relating them to performance (number of pigs produced, ADG or FE) but the recent appearance of electronic feeding systems allowed to understand the feeding behavior of the sows when they can choose when and how much to eat rather than eating the offered feed.

Eating behaviors in gestating sows

Another noteworthy variable, which can be measured in pig farms, is the eating behavior of the sows. This behavior is usually monitored using an ear transponder with Radio Frequency Identification (RFID) that identify the individual animal at each visit to the feeder (Bornett et al., 2000). Thanks to these technologies, it is possible to collect enough information to characterize the eating behavior of individual animals. For example, most ESF systems present in the market recognize the individual sow using RFID transponders and feed her according to her specific feeding plan by adapted feeding curves.

Besides accurate feeding, the main feature of ESF systems is helping farmers to overcome the problems they face in groups-housed pregnant sows such as the competition between animals, stress (especially in gilts and submissive sows) and waste of feed. Moreover, ESF systems allow promoting the ideal body weight condition for farrowing, a reduction of the time spent on food, and above all the rapid detection of sows with deviated feed intake patterns which could be an indicator of disease. All these systems generate alerts when a sow is not eating but usually don't go beyond

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this. A recent publication (Iida et al, 2017) demonstrates that more subtle variations in gestating sows' feed intake can be detected related to their gestation losses since sows losing gestation any time visit fewer the feeder and tend to eat less. The same authors demonstrated differences in the patterns among parities and genetic lines. By the proper implementation of these algorithms, risky eating behavior can be detected in advance before the sows stop eating completely, where the situation will be likely more severe.

There are two major factors to be considered in group sows the appropriateness of the decision to every company. The first one is competitiveness for the feed. Group sows are hierarchical and pay continuous attention to feeding since, in this phase, feed is normally restricted. Any system that allows any sow to take a part of the feed of another sow can be defined as 'competitive'. This is one of the factors that usually generates more stress in gestation pens. The other factor is data generation since there are systems that do not generate data at all while others, mainly those electronic ones, generate data in a routine (despite that many times are not used at all). Based on these two factors, we can propose a qualification of the feeding gestation systems (Figure 9).
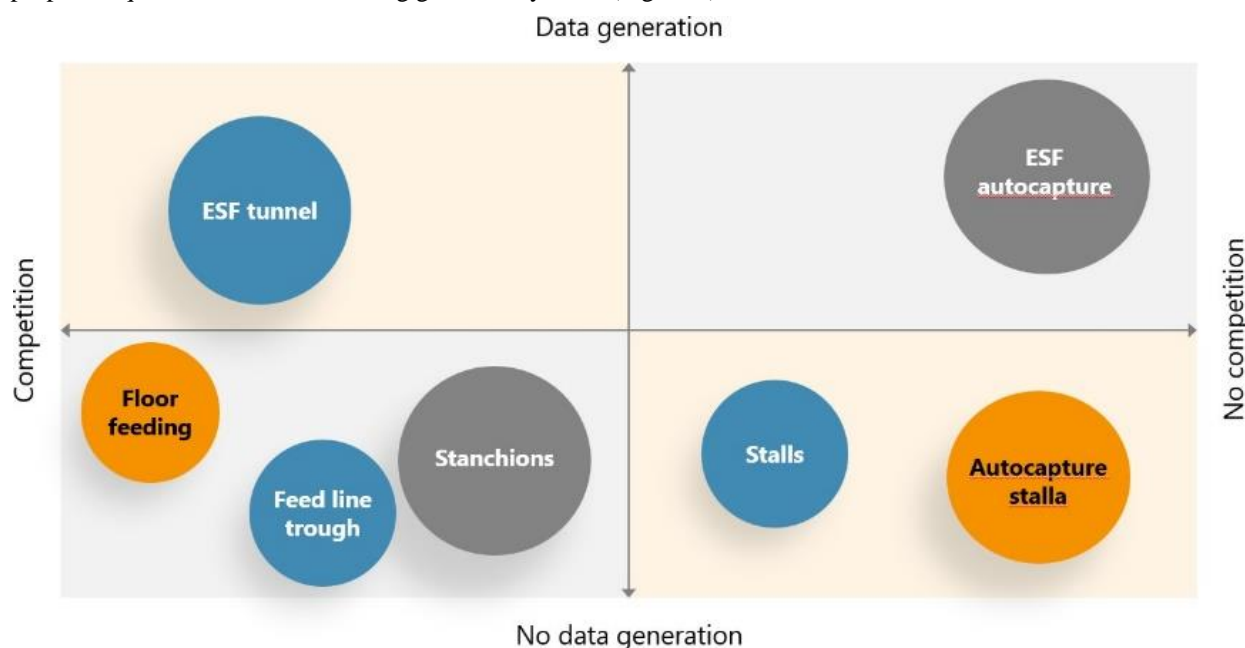

Figure 9. Types of group sows based on competition and data generation

It clearly seems that we need data to understand the modern sows, and its preferably that there is no competition among sows, and therefore it is expected a dominant position of these systems in the next decade. It is interesting also that beyond the averages, we can see intake patterns when the sows are allowed to choose to show mostly a nocturnal intake, finishing most of it at noon every day (Figure 10).
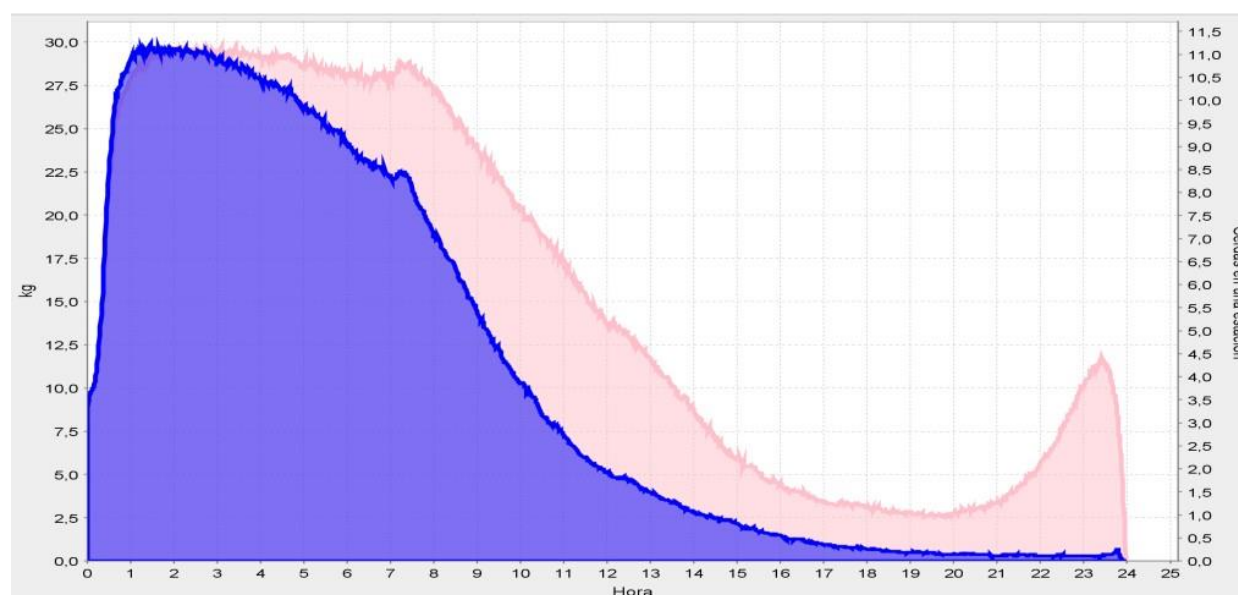

Figure 10. A 24 h feed intake pattern in a group of sows well managed

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Eating behavior in lactating sows

Lactation feeding has been traditionally provided by the farmers manually 2-3 times per day in most cases. Some of the equipment in the market just mimic this behavior, offering a pre-decided amount to the sows without physical human intervention. But there are some new systems that allow choosing the sow when and how to eat. These facts reveal a totally different situation where we can observe and analyze the eating behavior of the sow and react early to altered patterns. This is very relevant since deviation from the ideal feed intake pattern can impair the productive performance of sows. In this sense, Koketsu et al. (1996) categorized the lactation feed records of more than 25,000 lactating sows on 30 commercial farms in six patterns: 1) rapid increase in feed intake; 2) major and 3) minor drop; 4) low feed intake throughout lactation; 5) low intake during the first week then an increase in feed intake for the remainder of lactation, and 6) gradual increase. In this study, multiple regression analyses revealed that the average daily feed intake of sows during lactation had nonlinear or linear associations with the mains KPIs of swine production. It was demonstrated that sows either having lower feed intake throughout the complete lactation or during the first week or having a major drop, had longer weaning-to-first service interval and weaning-to-conception,and also had lighter litter weight at weaning than those with rapid increase, minor drop and gradual increase of feed intake. Until now, these results were very difficult or almost impossible to track in commercial farms, but recent ESF systems have changed this scenario. These feed intake patterns described by Koketsu et al. (1996) has been recently replicated in commercial farms, thanks to the use of the ESF system. Figures 11 and 12 show the graphs recently obtained in commercial farms of feed intake of three of the patterns previously described by Koketsu et al. (1996): the "normal pattern" (rapid increase of feed intake during lactation) and the two patterns which most deviate from the ideal intake pattern and can impair the productive performance of sows (major drop and low feed intake throughout lactation).

Combining these feed intake patterns with the scanner images of the ovaria during lactation when the oocytes are being generated for the next reproductive cycle, can be shown the relationship between the total intake/intake pattern and the number and quality of ovocytes to be fertilized after the weaning (image 1 and 2, courtesy of Antonio Vela-Thinkingpig).
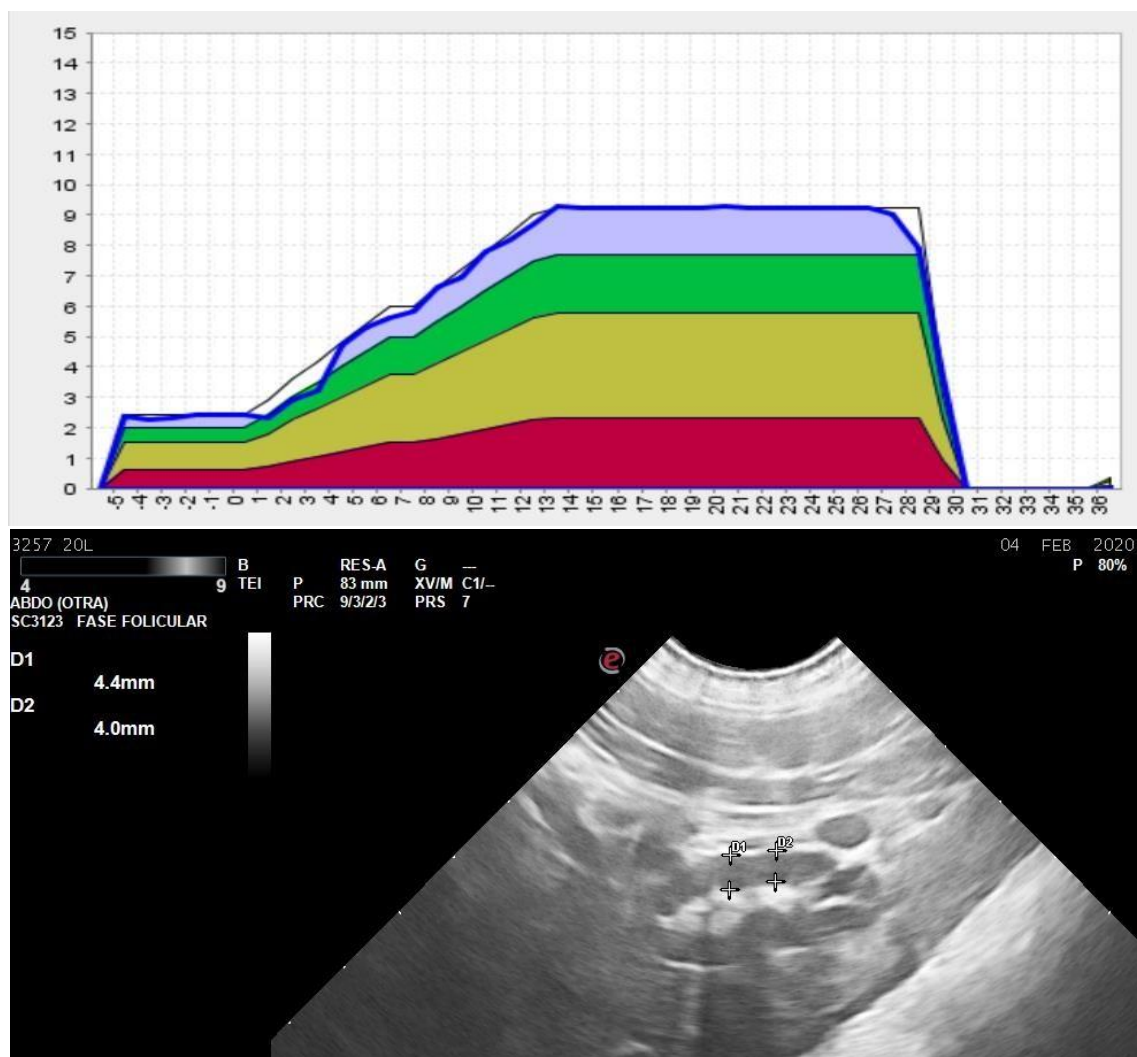

Figure 11 and Image 1. A sow with a good feed intake curve and a high number of oocytes of good size

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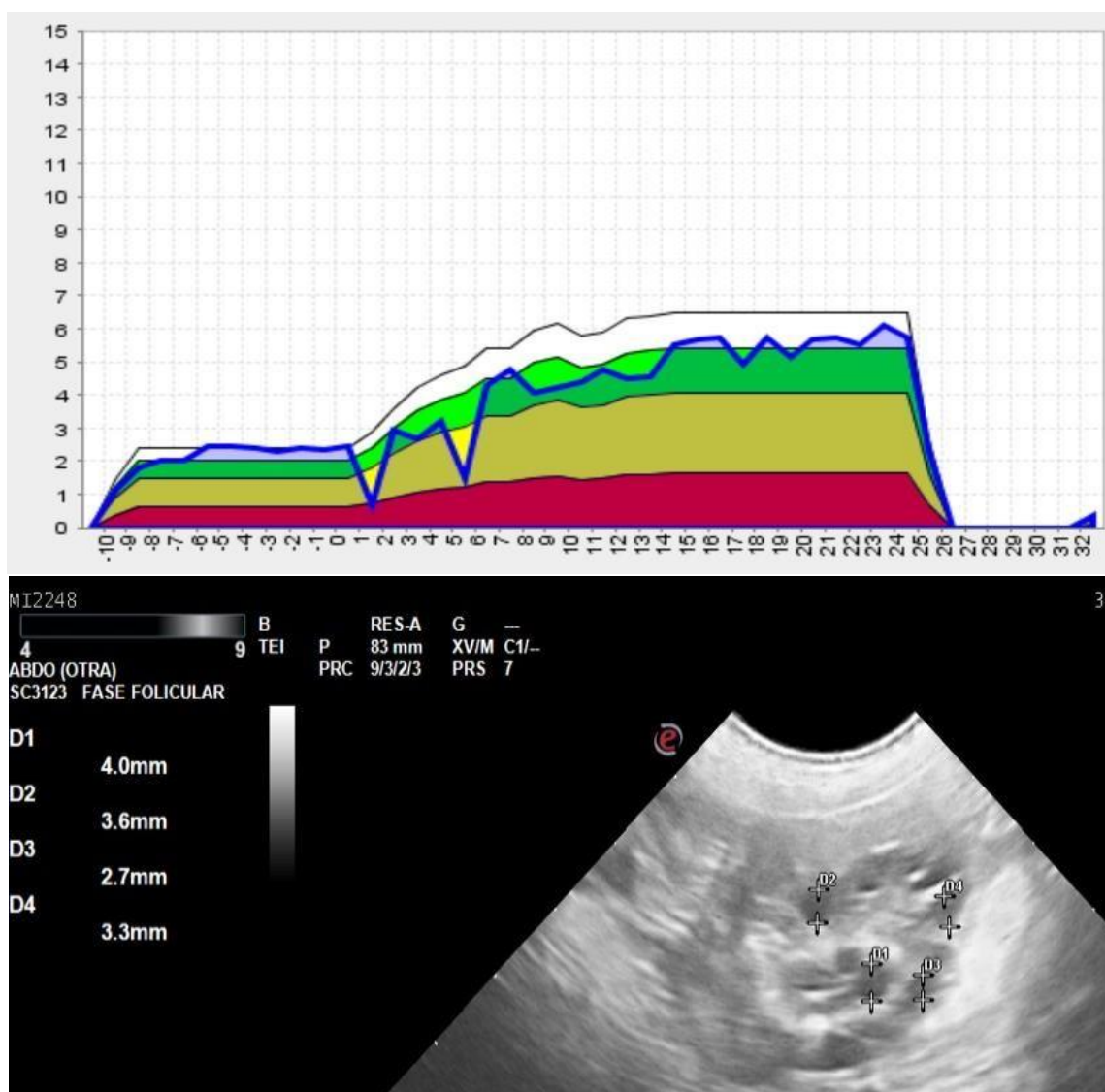congress – rio de janeiro – brazil

Figure 12 and Image 2. A sow with a poor feed intake curve and lower number of ovocytes that are smaller

This information allows us to monitor and react prompt and early to lactation problems (MMA, low intake related to pain) and start working in lactation to promote better performance in the next cycle.

Finally, and regarding growers and finishers nutrition, swine production systems have dramatically changed in the last three decades. Today main challenges look for maximizing feed efficiency while minimizing production costs and environmental impacts, mainly related to nitrogen and phosphorus excretion. Excretion of nutrients can be reducedby providing the animals with their required dietary levels, which also improves nutrient efficiency and reduces production costs (Pomar et al., 2015; Andretta et al., 2016a,b). Until now, this has been tried to be achieved by using different feeds changed as the pigs grow, normally considering the whole barn or pig batch for the change but without taking into account weight variability, sex or even health status, which unavoidably led to mismatches between the need and the nutrient offer.

In this context, precision feeding is a major breakthrough in pig nutrition and one of the most promising avenues to promote high-quality and safe pork, high animal welfare, and minimal impact on the environment. Precision feeding allows real-time off-farm monitoring and intelligent management of feeds and animals for improved economic efficiency, significant reduction of labor requirements, and early identification of animal environmental and health stressors thereby reducing the use of antibiotics. Utilization of precision feeding techniques in growing pig operations can significantly reduce production costs (>8%), protein and phosphorous intake (25%) and excretion (40%), and greenhouse gases emissions (6%) by increasing individual nutrient efficiency. This can be obtained under practical conditions by the use of new technologies from very simple to most sophisticated offering a mix of feeds for the whole barn, for individual pens or even individual pigs, being this last the most accurate one but also the most complex and costly to apply in commercial farms, appearing the feeding by pen mixing feeds as the most attractive in terms of cost-benefit for the industry (Figure 13).

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Figure 13. Feed mixed daily by pen in growing pigs.

In this scenario, a simple distribution by pen using sex (males -entire or castrated- and females) and size (small, medium or big) can generate a huge payback in terms of performance, costs savings and emissions reduction in a practical and manageable way with an attractive investment payback period (normally no more than 2.5 years) and return on investment (around 4:1) depending on the scenario of the cost of raw materials, the feed formulation approach and the slaughter weight (own data from an ongoing project not yet published). Many commercial companies are moving towards this direction in growing and finishing pigs as well, and the success will be determined to a large extent by the robustness, reliability and easiness for the farm staff, technicians and veterinarians. Once stablished, vets can monitor in real-time the total feed and the average disappearance-feed intake by pen, but also the pattern over time, promptly reacting to deviations due to health or management, ensuring the quality of the production and the expected flow and avoiding later major loses.

Health and antibiotics use

Health control has been well acknowledged as one of the pillars of our profession from long ago. In the last years has evolved in various directions, dealing mostly with enteric problems (bacteria and virus related), respiratory (porcine respiratory disease complex), but also with other systems affections (nervous, skin and legs) or even systemic (VanderWaal y Deen, 2018). Traditionally, data related to health control have been scarce. The most used KPI related is mortality percentage and normally used at the end of every nursery or fattening batch. Others like prevalence or incidence, despite being acknowledged as very important for most vets, are rarely used. On the other side, farm visits are becoming more difficult due both to animal health risks (both economic diseases like PRRS, mycoplasmosis or dysentery, among others) or catastrophic diseases like African swine fever. Until recently, it was very difficult to know and monitor real-time or almost real-time the health status in a farm and the way that antibiotics and other medicines are actually used but the recent appearance of SaaS solutions allow vets and managers to know more and better, controlling health better and providing better advise to their customers. Even laboratory data, usually underused, can be connected to farm health control, allowing at least:

1. **On-farm health, production and antibiotic use data**. Of what is actually observed and applied on the farm, generating real-time information on:
   o Prevalence and incidence of diseases of each batch/farm, being able to easily consolidate the data.
   o Treatments used by the workers (water/injections for antibiotics and various types of feed additives), and their impact on the observed diseases.
   o Of the correct dosage of each medicine at each time of rearing, being able to know their real use in mg per pig or per Kg produced, going beyond the 'purchased antibiotics'.
   o Weights, conversions and consumption of the feed used in each batch are also related to the three previous points.
   o Images of the lesions associated with the observations, necropsies carried out or the symptomatology of the animals in an orderly manner.

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2. **Laboratory analysis data**. These data are generally underused, beyond making decisions on the basis of the pdf received with the results of the samples sent or a simple summary of the raw data if the laboratory provides them. For example, it is not usually available:

- o   Prevalences of pathologies associated with specific viral and bacterial pathogens.
- o   Summary of antibiotic sensitivities to each pathogen
- o   Temporal evolution of the two previous points (by farm, by zones or in an aggregate way in the company), etc.

Its economic importance both for direct and indirect losses is also well described, and now the swine sector has bigger challenges than ever, including the growing and changing complexity of the infections, the appearance of new strains and the limitations in the use of antimicrobials the Deloitte report points health as one of the keys to support sustainability and control emissions.

Figures 14 and 15 show some examples of the system we are currently using in Spain to control health and antibiotics use.
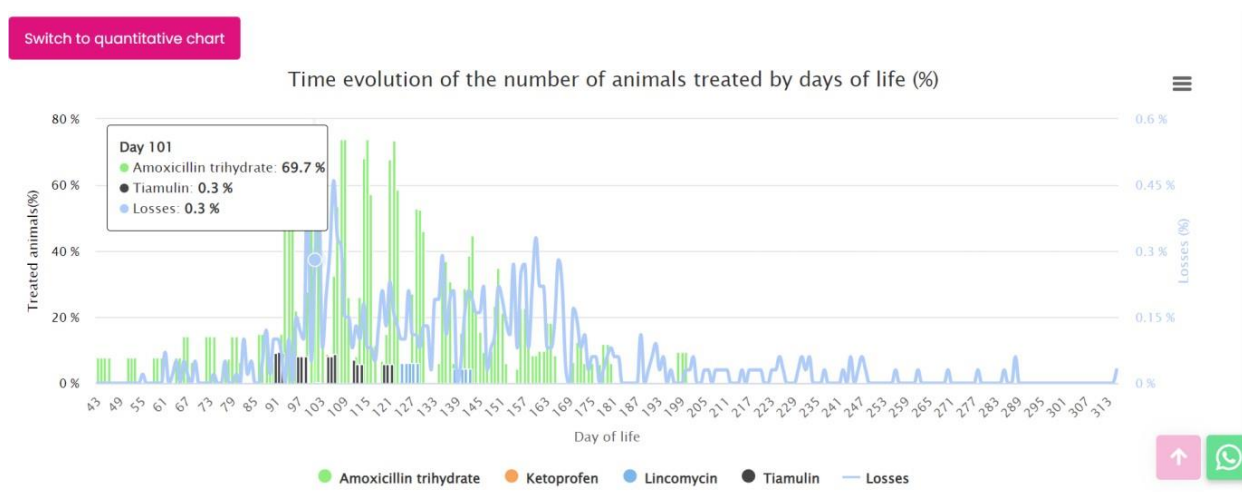

Figure 14. Time evolution of the number of animals treated by days of life (%)

Active ingredient	Dosage ratio	Situation	Total consumption	Total consumption / entered animal	Average day of life
Acetylsalicylic acid	1.0	Correct	2310001.6 mg	50.2 mg	37.0
Amoxicillin	0.9	Correct	1988273.1 mg	43.2 mg	34.5
Clavulanic acid	0.3	Underdosed	34380.5 mg	0.7 mg	34.9
Enrofloxacin	1.1	Correct	235795.0 mg	5.1 mg	41.0
Florfenicol	1.2	Overdosed	330615.0 mg	7.2 mg	38.5
Gentamicine	0.9	Correct	5120.0 mg	0.1 mg	42.2
Ketoprofen	1.1	Correct	150.0 mg	0.0 mg	97.0
Marbofloxacin	1.1	Correct	595921.0 mg	12.9 mg	41.7
Meloxicam	1.6	Overdosed	3941.8 mg	0.1 mg	36.8
Neomycin	0.1	Underdosed	44000.0 mg	1.0 mg	30.0

Figure 15. Antibiotics used, total consumption and dosing rate in a commercial farm

Biosecurity data

Biosecurity is known as the implementation of measures to reduce the risk of introduction and spread of disease agents (FAO, 2010) and can thus be divided into two aspects. External biosecurity relates to the prevention of pathogens entering a herd, while internal biosecurity prevents the spread of disease within a herd, mainly from older to younger animals (FAO, 2010). In this regard, biosecurity is an important aspect of preventing the transmission of diseases, thus improving health and reducing the need for antimicrobials (Laanen et al., 2013). Moreover, most diseases havea negative impact on the well-being of the animals and, consequently, on their productivity. Thereby higher levels of biosecurity lead to also improved the economy of the farmer.

Under our experience performing biosecurity audits in western/eastern Europe and Asia, it is quite frequent to get the same answer when we find mistakes' we always do well, but just today…'. And this is one of the crucial points, the exceptions. It is very common that farm staff knows, in general, the theory or the working principles they should follow, but they don't comply in every case. Following are presented some personal examples:

- **Responsibilities in biosecurity not clearly assigned** to anyone. Some farms have a comprehensive written protocol that is not put in practice because there is no one with the responsibility of its implementation and lack special trainingon biosecurity.
- **Mixing of personnel in common areas** (canteen) without changing boots or clothes.
- **Shower partially compulsory** because 'you are not going to take them a shower 4 times a day'
- **'I am special'**. Farm arranged by colors in clothes for every zone but manager wearing special and different ones without respecting rules just because 'I am the manager'
- **Public and private roads used shared** with no preventive measures at all.
- **'Creativity' in complying with the rules**. 'I only shower when I arrive in the morning'

Data were there wasn`t any

The human factor is known as of paramount importance in swine health and production since most of the tasks are performed by people. This includes how animals are managed in terms of animal care, feeding administration, application in practice of medicines and vaccines, follow-up of biosecurity protocols and accomplishment of farms' tasks and duties plans.

In this scenario of great progress in data management using different sources, our understanding of real human behavior in swine farms is lacking. Among others, this affects biosecurity and general farms' operations.

The assessment of the biosecurity level of a farm/group of farms is very complex due to the cross-sectional characteristics of the farm. Biosecurity affects all the processes that take place on a farm, animals, people, supplies, environment, etc. For this reason, it is essential to have an evaluation method that is as ordered and standardized as possible; only in this way details that can be of great importance will not be overlooked.

The first and most traditional method is biosecurity audits. These are based on surveys together with a farm visit and a final analysis of data collected. The limitations of this are clearly related to the subjectivity of data reported, sometimes even with the best will, but answers could be more related with a guess than with a fact. When visiting the farm this normally corresponds with the picture of what happens at that moment, which could match totally o partially with a fair image of farm facts and actions performed. Finally, follow-up is mostly based on the repetition of this process rather than in getting objective information about the recommendations agreed. Altogether, biosecurity audits appear at the best possible solution until now to understand and improve biosecurity standards but with a clear margin of improvement.

Although many diseases share common facts and approaches related to the biosecurity principles required, I will focus on one of the most common in the porcine industry; the porcine reproductive and respiratory syndrome (PRRS). This disease impairs swine health and is responsible for huge economic losses in the swine industry worldwide (Neumann et al., 2005). Infection with PRRS virus (PRRSv) is characterized by reproductive failures in pregnant sows, high pre-weaning mortality in piglets infected in utero and respiratory signs in both growers and finishers pigs (Done et al., 1996; Kranker et al., 1998; Rossow, 1998).

Numerous studies (Baysinger et al., 1997; Weigel et al., 2000; Mortensen et al. 2002, Otake et al., 2002a,b, 2004; Firkins & Weigel, 2004; Evans et al., 2008; Dee et al., 2009; Lambert et al., 2012) have described the routes which are involved in PRRSv transmission between and within herds, including the introduction of positive animals or semen, management of the quarantine for the newly introduced animals, as well as vehicles, aerosols, insects or contaminated fomites. Moreover, the marked genetic and antigenic heterogeneity of the virus, combined with its immune evasion strategies, inhibit the full efficacy of current commercial PRRS vaccines (Hu & Zhang, 2014). Therefore, PRRS control based only on the use of vaccination has often provided limited efficacy under field conditions (Geldhof et al., 2013). Hence, it is of paramount importance the implementation of good biosecurity measures to prevent the introduction of the virus into a farm but also to slow down its transmission within a herd once infected.

Nonetheless, most of the developed programs of biosecurity measures are based on scoring systems or survey forms. For instance, researchers from Ghent University developed a scoring system called Biocheck. UGent™ (Laanen et al., 2013; Postma et al., 2016) as a risk-based scoring tool to evaluate the biosecurity quality of pig herds. Another scoring system has been developed by the University of California-Davis (Disease Bioportal®) for the dynamic risk assessment and farms' benchmarking also based on surveys (Lin et al., 2013). In this line, Sternberg-Lewerin et al. (2015) developed a risk assessment tool for *B. hyodysenteriae* and *M. hyopneumoniae* considering the frequency of

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contacts, but it was only focused on external biosecurity. All these tools are based on values obtained through expert opinion panels; however, the perception of experts may vary depending on different circumstances; therefore, scoring systems based on perceptions should be adapted to each situation.

However, the development of objective systems to assess biosecurity is constantly evolving. In this regard, new solutions for digital biosecurity control are appearing in the market as objectives tools for the evaluation of internal biosecurity based on a system of control of the flow of internal movement of personnel on pig farms.

The new digital biosecurity system was based on two on-farm hardware pieces including *beacons* and readers. Each farm worker was given small *Bluetooth*^{TM} transmitters called *beacons*, which were required to wear all the time while they were within the farm facilities. Readers were installed and fixed at every access of every barn, including lockers and showers. These devices can detect *beacon* signals by proximity. Whenever a *beacon* was within a device's detection range, the device registered the *beacon* identity as well as the detection time and uploads the records to a cloud database.

Data collection and processing records from readers were sent to the cloud and processed, so the movements and routes of the farm's workers were computed. Each movement represented a route made by a farm worker from an origin zone to a destination zone. Thus, the system allowed the real-time monitoring of the farms' staff movements patterns. Figure 16 shows the map of readers set on a farm.
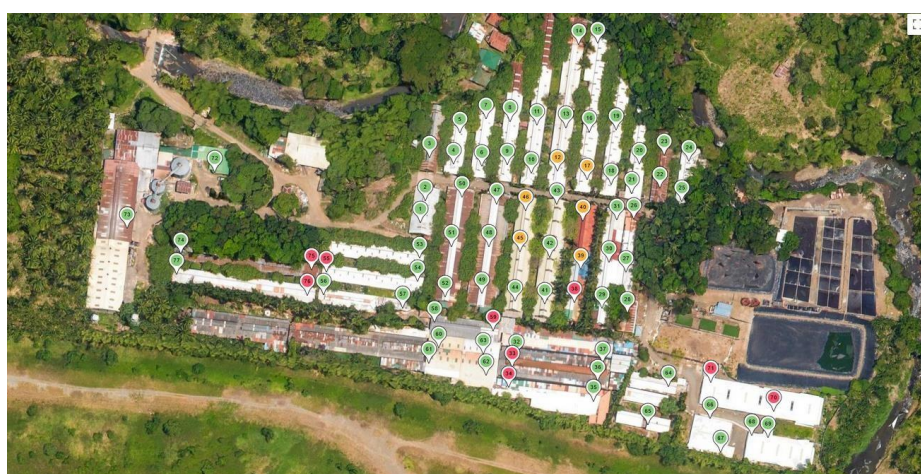

Figure 16. Map of readers for biosecurity movements control in a commercial farm

Díaz et al. (2018) have recently proved that the internal movements of farm staff are related to PRRVs incidence. First, personal farm movements were classified into Safe, Unsafe and Risk depending on which farm areas are being produced. Results showed that neither the percentage nor the total amount of both Safe and Unsafe movements were significantly different between the PCR Positive and Negative PRRS status groups, being Safe movements was always above 80% in the Negative PCR PRRS status group. However, both the percentage and the total amount of Risky movements were significantly smaller in the PCR negative PRRS status group. These results show a clear relationship between the total amount of Risky movements and the probability of a PRRSv outbreak on the farms (Figure 17).
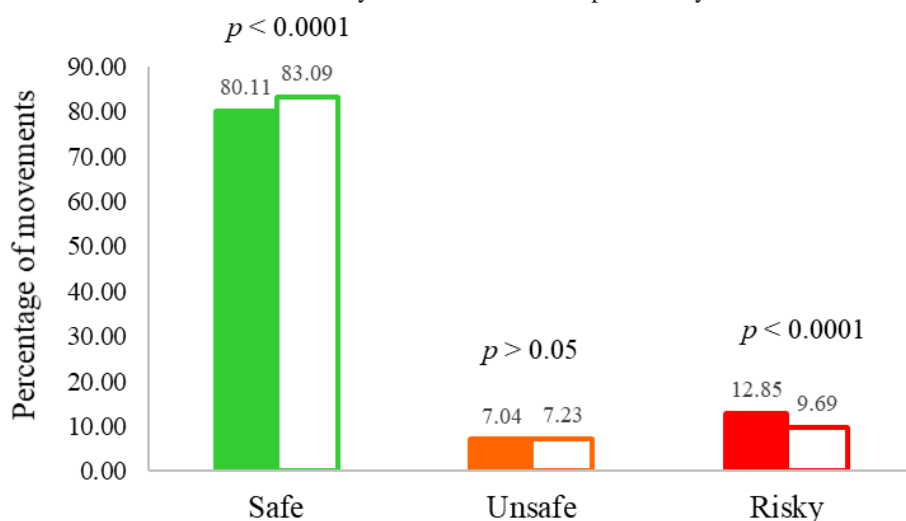

Figure 17. Average of percentages of Safe (green), Unsafe (orange) and Risky (red) movements before (■) and after (□) the training session considering the eight farms in which the system control movement was installed.

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Moreover, proper training can decrease risky movements and increase the safe ones (Figure 18).
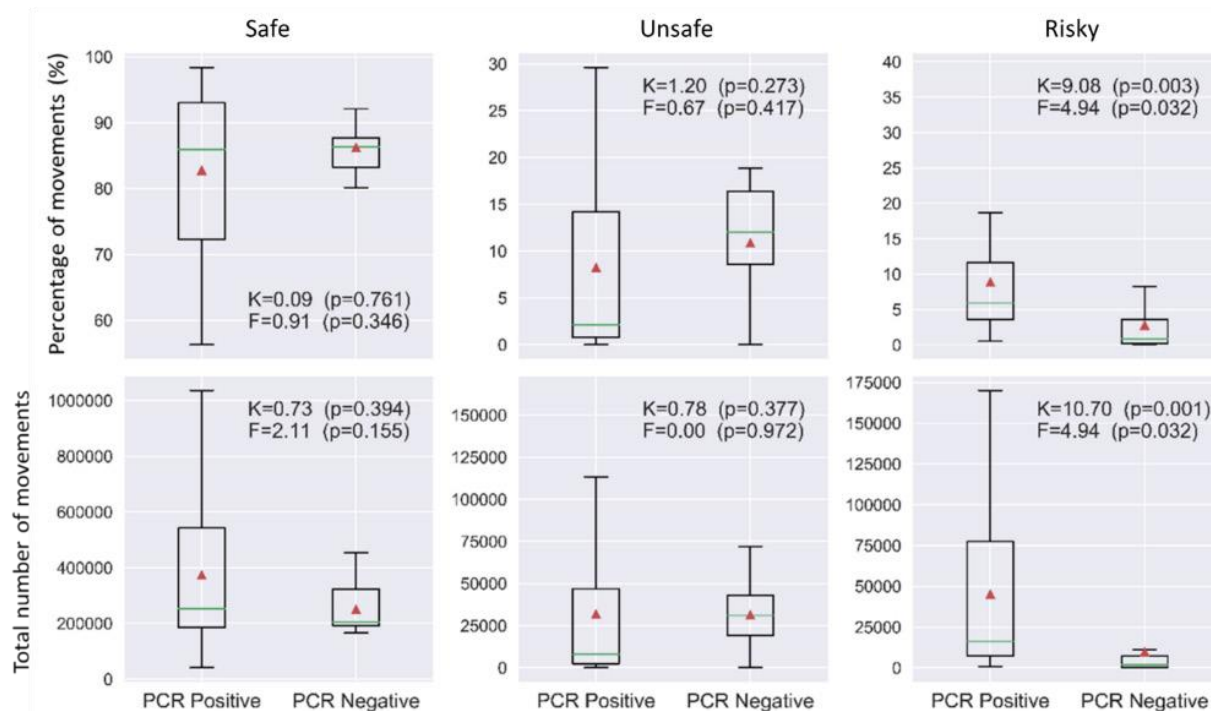

Figure 18. Comparison of percentages and totals of Safe, Unsafe and Risky movements between the PCR analytics positive and negative groups. The boxes extend between the first and third quartiles of the data for each group, and the whiskers extend between the minimum and maximum values. Group average is shown by the red triangles, while median lines are shown with solid green lines.

Operational data

Farms' operations must be performed every day more with a higher degree of accuracy to ensure expected results. Generating the expected number of pigs of a certain quality is a consequence of a good number of ordered actions adequately performed. Most of those tasks are performed by people and, generally, little is known about how it is really performed beyond of reports or checklists normally filled by farm staff or managers despite is not common yet in pig production, some companies are starting to use some techniques coming from other sectors to meet these objectives, including LEAN methodology, Six Sigma, Kaizen, Hoshin Planning or Balanced Scorecard in order to meet operational excellence. This can be described as a philosophy that embraces problem-solving and leadership as the key to continuous improvement. People are often unsure of how to approach the subject of operational excellence. It is a difficult term to define, and most people either find the topic to be too ambiguous or too broad to talk about. Operational excellence, however, is not a set of activities that you perform. It's more of a mindset that should be present within you and your employees. Now, you're probably thinking, "that sounds nice in theory, but how do I translate this into actionable steps?".

These technologies allow moving forward into that direction in an objective way, understanding better how human behavior influences farms' results. Recent work from Black et al. (2021) demonstrated a relationship between the movements and the efficiency in production in US farms (data not published). In her study, a five-hour increase in time spent in rooms by the manager was associated with an increase in one weaned piglet for every 10 litters (P =

0.01). In a second farm, an increase in time spent working in the farrowing rooms of approximately two hours per worker per week tended to increase the number of weaned piglets by one piglet for every 4 litters (P = 0.087). All together, better farm staff movements avoiding risks and dedicating the time where it is most required allowed a payback of at least 18 € per sow per year. Having control of this at a glance will be of great help to monitor those operations are performed as expected and facilitates the early personalized corrections (Figures 19 and 20).
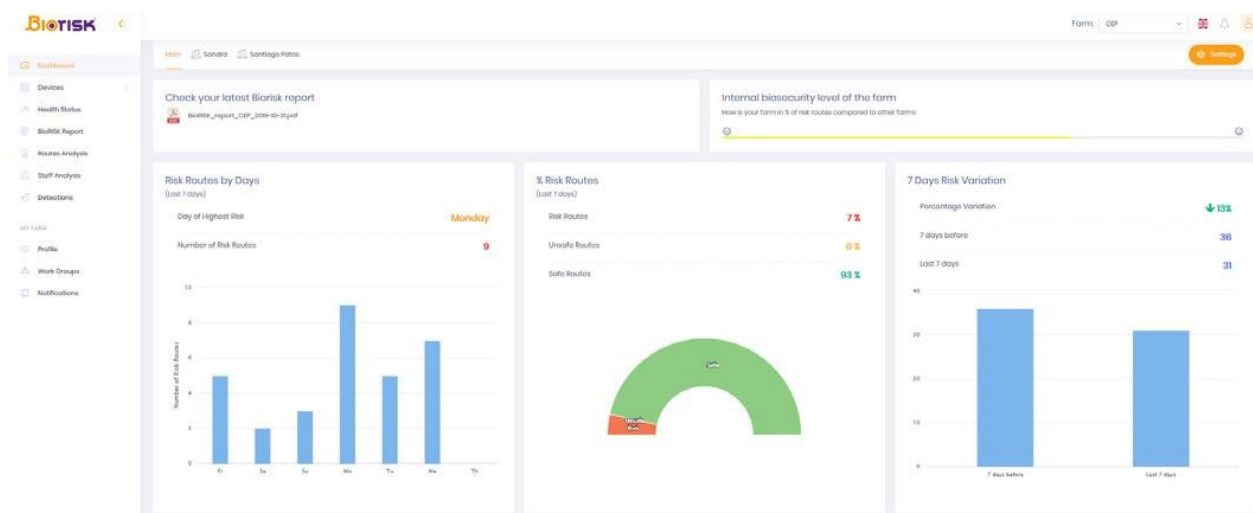
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Figure 19. Example of the dashboard to control movements related to internal risks in a commercial farm
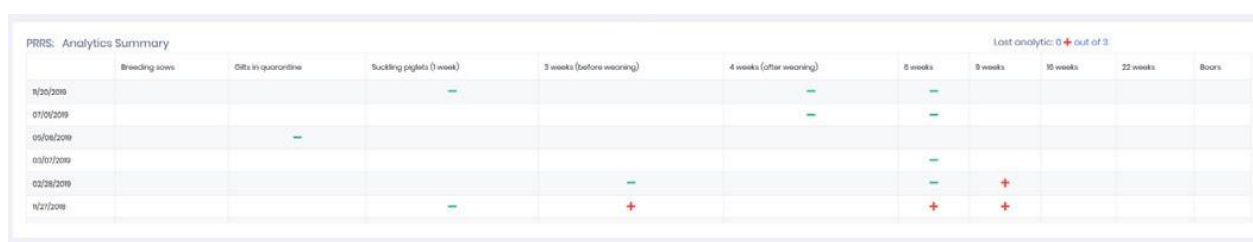

Figure 20. Over time evolution of the PCR + analytics for PRRS in a farm that controlled staff movements withthe digital system

Under our own experience, we have observed in farms worldwide some interesting facts that relate human behaviour and farm performance, both from visitors and farm staff:

1. **Football World Cup 2018**. In some farms, it was observed how on the days of national team games, the time spent by the workers in the mating area was reduced up to 35 %.

2. **Repeats of gilts**. In a Central America farm, we detected an unusually high percentage of repeats in gilts during the weekend. The time people spent in the mating area during the weekend was 40 % lower compared to the rest of the days and the multiparous area.

3. **Gilts development unit**. Wrong and forbidden farm staff entries during the quarantine period to gilts development units barn were detected which led to immediate preventive or corrective actions. Remarkably, nothing was informed from farm staff about these facts.

4. **Lactation feeding management**. When feeding sows manually in lactation it is recommended to space out the feed delivery to promote higher intake and less wastage. We have found evidence that the third feed delivery, expected in the afternoon, was brought forward considerably during the weekends.

5. **Holidays**. In Spain, we analyzed the movement and entry-exit patterns during the stay in certain areas of the farm. When one of the workers were on holiday and another covered the position, the pattern changed and the stays in that particular area were shorter and the entry-exit was more frequent. We believe that, due to the lack of habit of working in the area, the performance of the task was less efficient.

6. **Facilities**. In Spain, we also evidenced that the workers of the farrowing area spent more time in old barns to wean the same number of piglets that in new barns.

In the Figure 21 we can see the hours by farm zone and by day of the week of the farm staff.
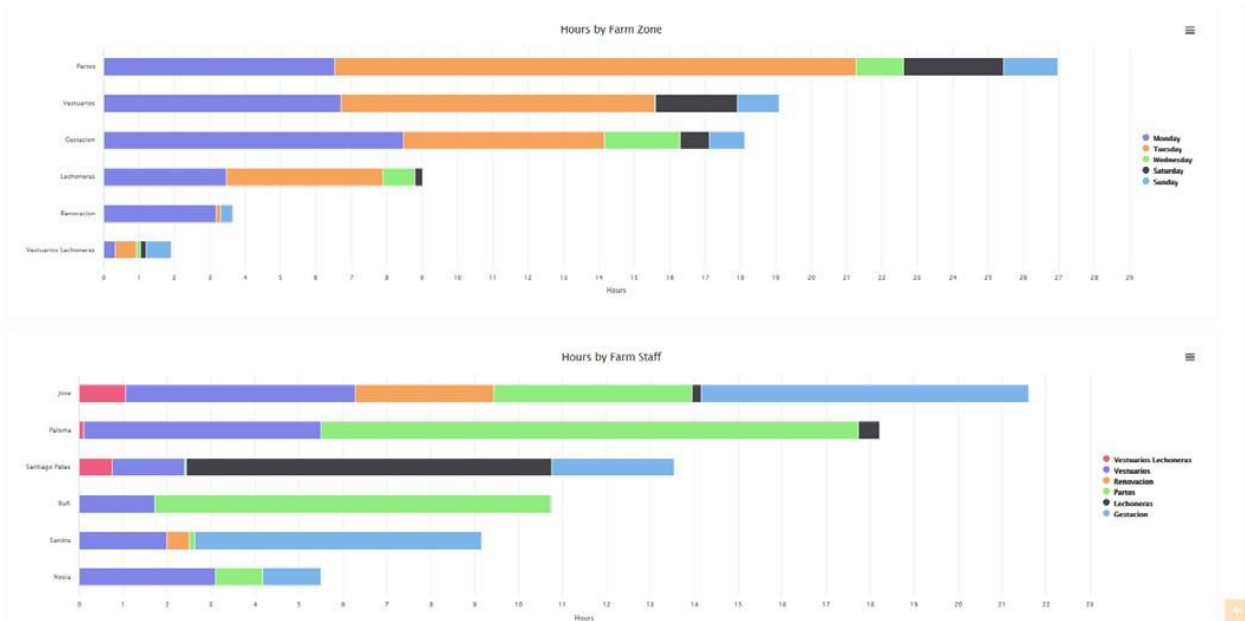
Figure 21. Metrics focused on hours spent by farm zone or by farm staff can be very useful to really understand the work performed and its pattern.

If every entry is a risk, it can be said that large companies, including this, are exposed to extreme risk. The system allowed to effectively control the flow of workers in farms responding to the restrictions of biosecurity established according to the company criteria, providing at the same time absolute traceability of all movements made and the possibility of immediate response. In this way, and in addition to the entry control, objective risk indicators are generated to manage and ensure biosecurity standards set. These new indicators are called "key biosecurity indicators" (KBI) and allow to keep an online and instantly updated record in the cloud (unlike traditional paper systems, which are difficult to understand and generate slow reactions). Figure 22 shows the trucks' movements and the risks related.
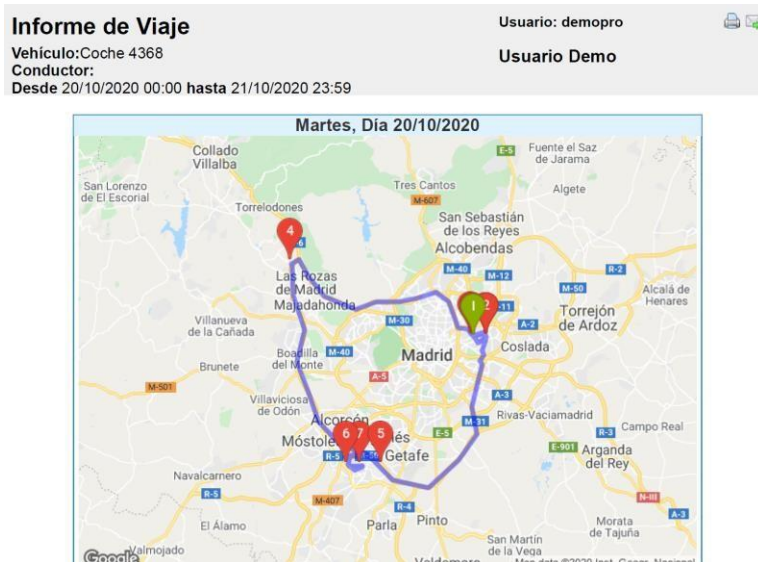

Figure 22. Trucks movements and traceability related.

This system can be used also with traditional disinfection centers but also with the new thermal disinfection ones, that can generate a controlled temperature in every point of the cage, supervised with a thermal camara and generating a token to be introduce in the system to ensure the later allowed routes for that truck (Figure 23).

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Figure 23. Thermal disinfection of a truck and data generated on the process

The system allows generating:
- Instant alerts to minimize damages, early control of its extent and prompt corrective actions.
- Easy monitoring of compliance with company standards.
- Detect those factors that generate more risk to the system and work specifically in its correction.
- Puts pressure on respecting the rules, since behavior improves where users are aware of being controlled.
- Perform biosecurity audits more objective, effective and focused, since they are based on data and not on checklists
- Design tailor-made training programs based on errors and not using generic ones.

The living labs scenario

Technology is and will be more present in commercial farms during the next decades. Its implementation in the routine will allow not only to achieve the benefits and consequences already mentioned but also to test different products (feed plans, additives, vaccines, breeding lines or other farm equipment) together with the right working protocols and experimental designs applied in farms combined with the routine of production. This, together with the role of farm advisors in the implementation of digital tools, will be another exciting option for swine vets since the generation of this information will be of paramount interest for the companies and one of the pillars for their sustainability and continuous improvement culture.

Conclusions

The swine production sector is becoming more professional than ever since it will be the only way to meet the objectives of efficiency and quality that support its competitiveness. Among other challenges, ensuring high biosecurity standards to keep farms free of disease and therefore improve productive efficiency and quality standards, mainly related with a low or minimum use of antibiotics, will be one of the key most important challenges in the next decade. Besides of this, promoting good or excellent operations will greatly help to achieve the objectives mentioned.

Digital technologies are very reliable and cost-effective will be a great new tool for the sector and in particular for vets that can do more and better consultancy work, without the mandatory need of visiting the farm, being able to understand better many factors that influence health and farm performance based on staff behavior, developing better solutions and deliver customized training to specific workers based on their behavior ad performance.

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Xenotransplantation: a history of pigs

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Introduction

Xenotransplantation is defined as the transplant with donor and graft being of different species, in opposite to allotransplantation in which all involved actors belong to the same species. Can be concordant when the species are relatively phylogenetically close (i.e., rat and mice) or discordant (i.e.: pig and human). But, why to think in something so technically difficult? To solve the shortage of organs; in Spain the donation rate is higher than 52 donors per million of population (dmp), the highest all over the world, but in countries as Germany or Brazil, the donation rate is quite lower (10 dmp and 17 dmp, respectively). And in Japan, the donation is lower than 5 dmp because religious reasons. As alternative to heart-beating death brain donation, several strategies have been designed to overcome this shortage of organs as living donation, donor in asystole, split liver, suboptimal donor and xenotransplantation. Among all, xenotransplantation is the only that remains as an experimental procedure, and in Spain the success of allotransplantation has resulted in a decrease of interest in this procedure. However, is a possible alternative yet, and recently the worldwide impact piece of news was the implantation of a kidney in a woman in death brain to assess the viability of the organ from a genetically modified pig (Weintrub, 2021), with 56 hours of correct function of the organ. This is a demonstration that pigs could become a source of organs for xenotransplantation.

In this article we are going to afford several aspects of xenotransplantation: the **history**, that sometimes is very prior to allotransplantation; the **look** for the best donor with the trials using primates, lambs or pigs up to the acceptance of the pig as the best option; the different **pigs** used in experimentation, from the first attempts using wild type (WT) pigs to the last genetically modified pigs by CRISPR-Cas9; the **results obtained in animal model** especially in the advanced pig-to-nonhuman primate (NHP) experiences; the **results in pig-to-human** model obtained during the last year, and the attitude towards xenotransplantation we can find in the population whom are going to decide in the future to accept or refuse an animal organ.

A little bit of history

The idea of humans with parts of other species is really ancient in the humankind collective ideology. We should only review the ancient prehistoric paintings with humans with head of bird in Lascaux caves in France, the Nordic histories of mermaid half human and half fish, the Egyptian pantheon with gods having head of ibis, jackal, cat, baboon, owl, falcon or crocodile, or the myth of minotaur and centaurs in the ancient Greek. A special example for all the people involved in xenotransplantation is the Lamassu (Sumerian: dlammař; later in Akkadian: lamassu), an Assyrian protective deity, represented as a hybrid of a human, bird, and either a bull or lion; specifically having a human head, the body of a bull or a lion, and bird wings (Figure 1). This chimera was selected as the symbol of the International Xenotransplantation Association (IXA).

But the complete image of a xenotransplantation is recorded at the 12ᵗʰ century in the ancient Hinduism tradition; the myth of Shiva, Parvati and Ganesha. Shiva (शिव in Sanskrit, lit. 'The Auspicious One') known also as "the destroyer" got married with Parvati (पार्रिती in Sanskrit, goddess of fertility) against her father Daksha's wishes. Ganesha (Sanskrit: गण॒ऀ॑) was born from Shiva and Parvati union, and there are different legends about the origin of his elephant's head. One of them tells that after marriage, Shiva and his forces waged war against the underworld beings. Parvati knew she was pregnant after Shiva leaves to the war, so he never knew that they were expecting a son. Paarvati lasted 15 years to come back home, and when he arrived, Parvati was having a bath and ordered to Ganesha to avoid the incomeat home from anybody. Then, Shiva found an unknown 15 years old youth prohibiting him entry in his own home. Hetore off Ganesha's head and threw it into the jungle. When Parvati discovered horrified what has happened, firstly crying required Shiva to restore the life of Ganesha and finally she threatened to destroy the universe if Ganesha wasnot resuscitated. Shiva sent their forces to look for the head into the jungle, and being impossible to find it, he promisedto put the head of the first animal he will find in the jungle; and the head of the elephant was joined with the headlessbody of Parvati's son, thus reviving him. It is the first complete idea of a xenotransplantation: a human being losing amember or an organ, which is restored with the organ of other species.

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Dr. Reemtsma, one father of the modern xenotransplantation, pointed out that possibly one of the earliest examples of xenotransplantation was the attempt by Daedalus and his son, Icarus, to fly across the sea from Crete to mainland Greece with the help of bird wings attached to their arms. Icarus failed in the attempt, and Reemtsma put this forward as a possible case of hyperacute rejection (very rapid rejection of the graft), though he thought it was more likely to be related to failure of a thermolabile adhesive. However, Daedalus successfully made the journey, providing this pair with an enviable 50% success rate (Cooper, 2012)

Deschamps et al. (2005) offered a really complete history of the first xenotransplantation recorded. In the prehistory of the transplants, only tissues and cells can be grafted since the vascular anastomosis was invented in 1907 by Alexis Carrel. So, the first xenotransplantation was made with blood, bone, skin or testicle. The xenotransfusions were assayed and recorded since 17th century in different countries using lambs, calves and sheep in first instance as donors. The last experience in humans recorded occurs in India, using blood from pigs and during a surgery Dr. Dhaniram Baruah delivered more than pig half a litter of pig blood to a patient (Hoffman, 2000). Since it was not a legal procedure never was scientifically communicated). Among tissues used in xenotransplantation, there are records of dog-to-human bone xenotransplantation in Iran, Afghanistan and The Netherlands in the 16th and 17th centuries, pancreas of sheep in United Kingdom in the 19th century or testicles and ovaries form ape in France at the early 20th century.
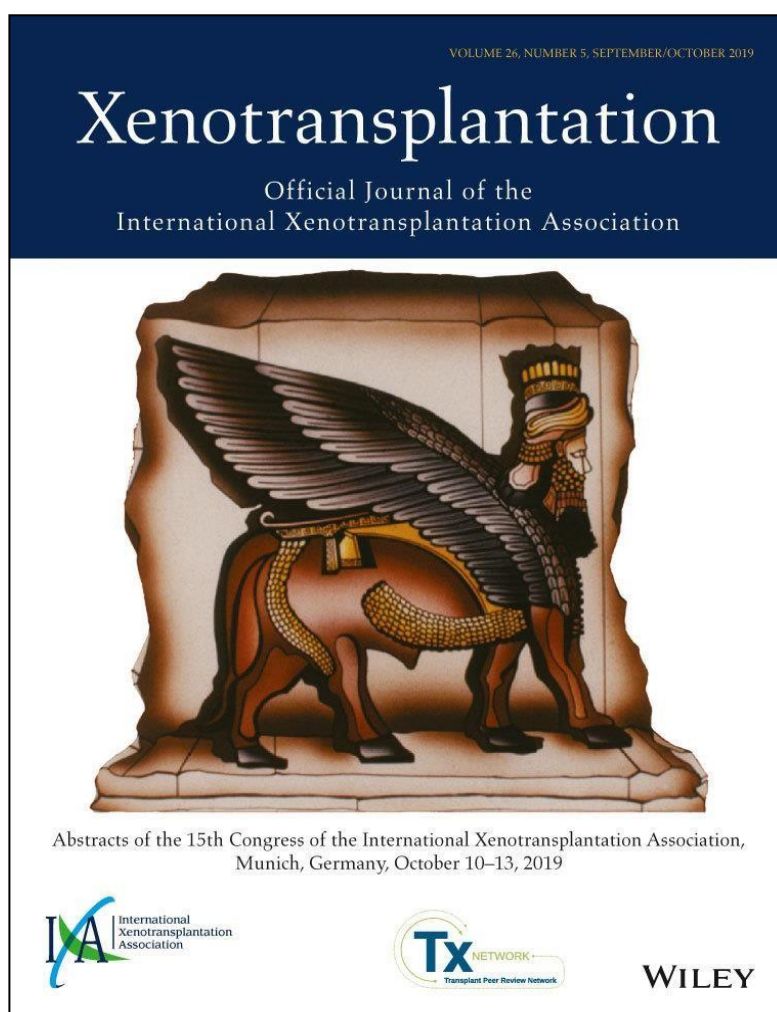

Figure 1. Lamassu, the image of the International Xenotransplantation Association as appears in the Xenotransplantation Journal volume devoted to the abstracts of the 15th Congress of the IXA at Munich in 2019

After vascular anastomosis development starts the history of solid organ xenotransplantation, and except for lung, there are experiences recorded with all solid organs; heart, kidney and liver as the main protagonist of this history.

The first kidney experience recorded was in 1905; with the insertion of rabbit kidney's slices in a child in France with 16 days of survival. After this, during all the 20th century has been records of kidneys xenotransplantation using as donors pigs (Jaboulay, France), monkeys (Unger in Germany), lambs (Nuehof in USA), baboons, Rhesus macaque and chimpanzees (Reemtsma, Starzl and Hume in USA or Traeger in France). Since 1966 up to 2021 there were no communications of xenotransplantation in models involving humans.

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The xenotransplantation of hearts started three years before the first allotransplantation by Barnard in South-Africa (1967). The first experiences involved chimpanzees (Hardy in USA) or pig (Ross in UK), and also there were experiences using chimpanzees and baboons, being especially remarkable the baboon-to-human xenotransplantation occurred in 1984 in USA; Stephanie Fae Beauclair also known as Baby Fae was born with hypoplastic left heart syndrome, incompatible with life. When she was 11 days old, Leonard Bailey xenotransplanted her with a baboon's heart and the graft survived 21 days up to a complete failure due to ABO incompatibility (https://en.wikipedia.org/wiki/Baby_Fae, Bailey et al., 1985). In that age the piece of news on Baby Fae opened the news bulletins all over the world during days, because the procedure opened a gate to the hope for thousands of children needing for a transplant and affected by a very deep shortage of organs because a very low infant mortality in western countries. Interestingly, this procedure did little to advance progress in xenotransplantation, but it did draw public and medical attention to the dearth of deceased human organs available for infants in need of a transplant. Following the procedure, particularly with the immense publicity associated with it, the situation with regard to donation of organs from infants became very much improved, and Bailey went on to develop an extremely successful cardiac allotransplantation program in infants and children at Loma Linda University. The last recorded experience occurred in India also developed by Dr. Baruah using the heart of a pig in a 32 years old man and surviving 7 days. As occurred with the xenotransfusion this procedure never was published in scientific media.

The liver xenotransplantation started 6 years after the first allotransplantation. In 1960, Thomas Starzl, the first surgeon doing a liver allotransplantation, reported a xenotransplantation using a chimpanzee's liver in a 28 months old child with a survival of 9 days After that, experiences have been recorded in France and USA, using baboons and chimpanzees as donors with survivals up to 70 days (Starzl in USA, 1992). There is only one record using a pig liver in USA by the team of Makowka, in a 26 years old man and a survival of 24 hours.

All the experiences of the pioneers demonstrated that xenotransplantation was not going to be an easy task, and at the early 90's of the 20th century stopped any experience using humans since the regulations became harder and the health authorities around the world implemented clear requirements of safety and efficiency demonstration before proceed to a new experience in humans. Moreover, the shadow of the possibility of pathogens jumping between species and the possible impact on public health pushed to stop the experiences that had been developed during the 60-90's years.

Which is the better specie as donor?

Considering the brief history of xenotransplant stated in the former section, is easy to see that non-human primates were considered very early as the best choice as donors. The main reason was the idea that the phylogenetic closeness to human would avoid the immune rejection. In fact, in the first two decades of 20th century, farms of monkeys were stablished in different countries to be organs source, being noticeable the case of Dr. serge Voronoff who built a farm of monkeys in France in the 1920's (https://en.wikipedia.org/wiki/Serge_Voronoff). The idea of Voronoff was grafting monkey testicle tissue on to the testicles of men for purportedly therapeutic purposes. However, very soon was clear that the closeness was not enough to avoid all the immune events related to rejection immediately after engraftment. Moreover, there are several added inconvenient that make primates bad donors:
-   The closest species (chimpanzee, gorilla and orangutan) are endangered of imminent extinction, so there is a social and ethical conflict.
-   But the most important reason to avoid primates as donors it that most of primate species have specific herpes and retrovirus which can jump easily among species in the same order (humans are primates) and this became an insurmountable obstacle when it was clear that virus as HIV1 could proceed form the SIV1 of the chimpanzee.
-   The reproductive success in captivity is reduced and the time needed to get the adequate size to be donor can rest years. Even when the reproductive rate in species as baboon is high (more than1 born baby er year in captive colonies), the growth rate in all NHP is really low.

So, decades ago there is a scientific consensus about the suitability of pigs to be donors in xenotransplantation. The advantages:
-   Very high reproductive rate, with more than 40 piglets weaned per sow per year. Of course, this is not the expectable reproductive rhythm in genetically modified animals, but in any case, the number of animals produced by a pair of breeders exceed 25 per year in the worst of the cases.
-   Speed of growth: 4 months to produce the liver for a 90 Kg human being in normal pigs. Attending to the

    weight recorded in the last heart xenotransplantation to human (January 2022), the pig weighted 120 Kg at one year of age. Of course, is not the growth rate recorded for commercial crossbreds, but in any case, we could have an adequate pig for liver transplantation in 6 months.
-   The specie with the most developed reproductive, diagnostic and preventive techniques. Routinely, thousands of diagnostic procedures are developed every day all over the world. And most of the efforts in swine

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medicine are focused in prevention. The possibility to produce SPF animals is a reality in pigs, so the infectious risk is lower than in other species.

- The absence of ethical conflict since more than 3 million pigs are scarified all over the world for meat production. However, we have to take into account that in the current environment, any usage of animals is going to produce, potentially, ethical concern and even complains from animalist groups as PETA (People for the Ethical Treatment of Animals), describing the last heart and kidney xenotransplant communicated as "*a stunt of junk science*" and affirming that "*animal-to-human transplants are unethical, dangerous and a tremendous waste of resources that could be used to fund research that might actually help human*" (Prater, 2022).

- Moreover, we know that the donor has to be genetically modified to overcome some of the events involved in rejection, and again, the pig is the specie with the highest level of genetic engineering and in vitro reproductive techniques, both necessary for genetic modification of donors.

The barriers to the xenotransplantation

The difficulties that the xenotransplantation have to face out are several:

- Technical barrier: the surgery obstacles; how to connect the vessels and ducts of the pig liver to one human (or primate in the experimental model). Apparently, both in the orthotopic and in the heterotopic engraftment the technical barrier has been overpassed.

- Immunological barrier: is the most important since is the first step. The immune rejection occurs even in allotransplantation, so it is expectable to have a more severe immune rejection in xenotransplantation. The immune events after xenotransplant are: hyperacute rejection (HAR), acute vascular rejection and cellular chronic rejection. The HAR is triggered by a simple sugar present in the endothelia surface of pigs (and New World Monkeys) but no in the Old-World Monkeys (including humans). This major xenoantigen is galactose-α1,3-galactose (α-Gal), which is expressed by α1,3-galactosyltransferase (α1,3GalT; also known as GGTA1) (Good et al., 1992). The problem is that the antigen, galactose-α 1,3-galactose commonly known as α-gal is very simple and is present in a wide range of bacterial walls; so, we are sensitized against the antigen days after births, and have antibodies against it (Galili et al., 1988). When we engraft an organ having α-gal triggers a complement activation; a very simple but destroyer immune action. If the organ survives the HAR, there is a reaction based on antibodies more elaborated (not anti- α-gal) and in cells (NK and T CD8+). And finally, a cellular chronic rejection, very similar to the produced in allotransplantation.

- Physiological barrier: there are a lot of unsolved questions regarding this barrier. We should take into account that the life hope of a human in 85 years and in the pig is 15 years. So, it is known the aging of a pig's organ inside a human. Moreover, we know that some pig hormones and growth factors does not works well in primates. In example, the pig erythropoietin is not able to stimulate the bone marrow of monkeys or baboons resulting in a deep thrombocytopenia with alteration of coagulation.

- Infectious barrier: the chance to produce a jump of pathogens from a pig to an immunosuppressed human is a possibility. This is the reason why we produce SPF pigs to be donors The other risk that threatened the xenotransplantation experimentation in the 90's is the Porcine Endogenous Retroviruses (PERVs); viruses that inserted their genome in the pig cell's thousands of years ago and does not produce alive virus, but potentially could be activated and produce virus. We should forget that since decades ago pig material is used for cardiac valves, in example, without any case of PERVs activation. Moreover, we should not forget that the human himself can have up to 500,000 copies of Endogenous Human retroviruses, and recently a theory stablished that they could be indispensable for the evolution from the first hominids up to the modern *Homo sapiens.*

Genetic modification of pigs as donors

The idea of the need for genetic modification arise at the earlies 90's. In fact. Cozzi and White (1995) defined clearly the modification that could help to progress the xenotransplant up to clinic level. The modifications have to be focused on the different barriers to the xenotransplant, especially against the immunological barrier, and today is well accepted that HAR can be completely avoided, and other alterations as coagulation disorder, ischemia reperfusion injury, innate immune response, cell-mediate rejection and the incompatibilities associated with organ function can be partially avoided (Gock et al., 2011). The different chances are:

- Modification of the α-gal antigen: we can apply two different strategies; introducing the gene for the α1,2 Fucosil transferase which convert the α-gal in a B blood group reducing then the immune reaction (only the A humans would have HAR), and to knock-out the α1,3 galactosyl transferase which avoid the insertion of one galactose and produce a smaller and not immunogenic antigen. The first animals are known as HT pigs and second as GTKO pigs.
  - Modification of other surface sugars: not only α-gal can be involved in rejection, another such as N-Glycolylneuraminic acid or Sda (involved in some not ABO blood groups)

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-   Modification conducting to control of complement. Since the HAR reaction is based on the complement activation, the control of this immune mechanism is a major target. The most popular strategy assayed is the transgenesis for complement regulatory proteins. These proteins prevent the accidental activation of complement, are control points for the complement cascade. The complement regulatory proteins devoted a homologous protection; the swine proteins do not protect against human protein, and then to introduce transgenes for these human proteins would protect the pig organs. The principal proteins are Decay Accelerating Factor (DAF or CD55), CD46 and CD59. The two first are C5-convertase controller and the last avoid the poly-C9 formation.
-   Other modifications

A summary of the most used modifications appears in the Table 1.

Table 1. List of genetic modifications

Immune rejection			
Abbreviation	Gene name	Function	Reference
GTKO	α1,3 galactosyltransferase KO (GTKO or GGTA1 KO)	Deletion of αGal epitope	Phelps et al., 2003
CMAH KO	CMP-N-acetylgalatosaminyltrasnferase KO	Deletion of Neu5Gc epitope	Deug-Nam et al., 2013
β4GalNT2 KO	β-1,4N-acetylgalactosamunyltransferase KO	Deletion of SDa epitope	Estrada et al., 2015
Neu5Gc KO (CMAH)	N-glycolylneuraminic acid	Deletion of CMAH, the enzyme responsible of Neu5Gc synthesis	Burlak et al., 2014
hCD46 (MCP)	Human membrane cofactor protein transgene	Inactivation complement factor C3b and C4b	Diamond et al., 2001
hCD55 (DAF)	Human decay accelerating factor transgene	Acceleration of complement decay	Coozi and White, 1995
hCD59 (MAC-IP)	Human membrane attack complex C5b-9 inhibitory protein transgene	Inhibition of the complement membrane attack complex C5b-9	Fodor et al., 1994
Coagulation disorders			
hTBM	Human thrombomodulin transgene	Activation of protein C	Petersen et al., 2009
hTFPI	Human tissue factor pathway inhibitor	Antagonize the function of tissue factor	Lin et al., 2010
hCD39	Human ectonucleoside triphosphate diphosphohydrolase-1 transgene	Anticoagulation and anti-inflammatory	Wheeler et al, 2012
ASGR1 KO	Asiaglycoprotein receptor 1	Decreases human platelet phagocytosis by pig sinusoidal endothelial cells	Paris et al., 2015
Inflammation regulation			
hA20	Human tumor necrosis factor alpha-induced protein-3 transgene	Inhibition of NF-kppa B activation and TNF-mediated apoptosis	Oropeza et al., 2009
hCD47	Human integrin associated protein transgene	Regulation of macrophage activation and phagocytosis	Tena et al., 2014
CTLA4-Ig	Cytotoxic T-lymphocyte-associated protein 4-immunoglobulin transgene	Cellular immune response: inhibition of T-cell costimulation via CD86/CD804	Wang et al., 2015
CITA-DN	Cytotoxic T-lymphocyte-associated protein 4-immunoglobulin transgene	Suppression of T-cell activation	Hara et al., 2013
hHO1	Human heme oxygenase 1 transgene	Antiapoptosis, cytoprotection anti-inflammatory	Petersen et al., 2011
Infectious risk			
PERV inactivation	Porcine endogenous retrovirus inactivation	Xenozoonosis	Niu et al., 2017

The first attempts to produce transgenic pigs was so random; the technics available 20 years ago consisted in introduce DNA constructions in oocytes, allowing to implant in the nucleus, in a random way and then explore the expression of the transgene. Using this ancestral technique were produced hCD55, hCD59, hCD46 or hTransferase pig which were pioneers in the pig-to-nonhuman primates (NHP) models. In fact, there were survival of months for heart and kidney xenotransplants using these animals (Table 2). But it was not enough. Then the knock-out techniques arise and allows to produce animals with gene silenced, as the GTKO pigs, knockout for α1,3 galactosyltransferase gene; and then producing pigs without α-gal on endothelium surface. This antigen is critical for HAR, so the first and more destructive immunological event can be avoided.

But the real jump forward in xenotransplantation came with the age of the CRISPR-Cas 9 technology. This procedure opens a new universe of possibilities. In fact, the major difficulty of the genetic modifications was that the addition of modifications should not result in success. The gene editing technology allows to make accurate modifications, with

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more possibilities of changes. In fact, nowadays CRISPR-Cas 9 not only allow to obtain animals with a lower immunoreactivity, but also is able to reduce some infectious risk, inactivating the PERVs (Güell, 2020). Briefly, this method is based in the CRISPR (clustered regularly interspaced short palindromic repeats), a family of DNA sequences found in the genomes of prokaryotic organisms. Using probes that can identified this CRISPR and together with enzymes such as Cas9 (CRISPR-associated protein 9) can be edit genes, switching-off them, or introducing changes that could humanize the pig genes. In the next section it will be clear that the results in animal models from 2014 are qualitative and quantitative better, and the main reason are the procedures to obtain the donors.

Results in animal models

Obviously, the most of research has been developed in animal model. The most frequently used models had been rat-mice, rat-to-guinea pig, and when the donor is a pig, pig-to-baboon and pig-to macaque (Rhesus and Cynomolgus). The success of xenografts is listed in the Table 2.

Table 2. Selected experiences in xenotransplantation in pig-to-NHP model. The references included in the table are available under demand.

Organ	Model	Main features	Authors
Liver	Pig-to-baboon	Pig: hCD55 and Htransferase N= 18 Survival = 8 days	Ramirez et al, 2001, 2005 (Spain)
	Pig-to-baboon	Pigs: GTKO/hCD46 N = 8 Survival =7 days	Ekser et al., 2011 (USA)
	Pig-to-baboon	Pigs: GTKO Survival = 25 days.	Sah et al., 2016 (USA)
	Pig-to-baboon	GTKO. Survival = 29 days.	Sah et al., 2019 (USA)
Heart	Pig-to-baboon	CD55 pigs. Severe immunosuppression. Survival= 4–139 (Median=27)	Houser (2004) Kuwaki (2004) (USA)
	Pig-to-baboon	Pigs transgenic for CD46 Baboon to 10 baboons. Included sever immunosuppression. Survival 56–113 (Median=76)	McGregor (2005) (USA)
	Pig-to-baboon	GTKO (MSw). Survival= >16–179 (Median=63)	Kuwaki (2005), Tseng (2005) (USA) Hisashi (2008) (USA) Shimizu (2008) (Japan)
	Pig-to-baboon	GTKO/CD46. Survival=36-236 (Median=71)	Mohiuddin (2012) Corcoran (2010) Horvath (2010) (USA)
	Pig-to-baboon	GTKO/CD46/TBM. Survival = 77-380	
	Pig-to-baboon	GTKO/CD46/TBM. Survival=195	Mohiuddin (2013, 2014) (USA)
Kidney	Pig-to-Cynomolgus macaque	CD55. Survival=78 (39) and 35 (Median=13)	Langin et al, 2018 (USA) Cozzi et al., 2000 (Italy)
	Pig-to-Cynomolgus macaque	WT. Survival=4-287 (24)	Loss et al (2001) (USA)
	Pig-to-baboon	GTKO/CD55. Survival =4-68 (32)	Yamada et al (2005) (Japan)
	Pig-to-baboon	GTKO/MsW. Survival = 18-83 (Median=49)	Griesemer et al (2009) (USA)
	Pig-to-Rh macaque	GTKO/CD55. Survival =499	Kim et al., 2019 (USA)
	Pig-to-Cynomolgus macaque	GTKO/CMAH KO/β4GalNT2 KO. Survival=316	Ma et al, 2022 (USA)
Pancreas	Pig-to-Cynomolgus macaque	WT. Survival=11	Rijkelijkhuizen (2000a) (The Nehterlands)

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	Pig-to-Cynomolgus macaque	WT. Survival=>158	Hering (2006) (USA)
	Pig-to-Rh macaque	WT. Survival=409	Rogers (2007) (USA)
	Pig-to-Cynomolgus macaque	CD46. Survival=396	van der Windt (2009) (USA)
	Pig-to-Cynomolgus macaque	WT. Survival=224	Vériter (2013) (Belgium)
Cornea	Pig-to-Cynomolgus macaque	WT. Survival=60-180 (165)	Amano (2003) (Japan)
	Pig-to-Rh macaque	WT. Survival=>180	Li (2011) (China)
	Pig-to-Rh macaque	WT. Survival=194-398	Choi (2011) (South Korea)
	Pig-to-Rh macaque	WT. Survival=421	Choi (2013) (South Korea)
	Pig-to-Rh macaque	WT. Survival=933	Choi (2015) (South Korea)

The heart and kidneys model uses to be heterotopic; preserving the heart of the graft, and placing the engraftment in a non-natural territory of the body, and normally is assessed the survival of the organ. Results quite interesting observe the forward jump in survival since 2014, the year of the gene edition invention. Since that moment, the survival for heart and kidney has increased in 2 to 3 folds.

Also is interesting the case of cornea and pancreas islets. For both organs, the donors are WT pigs, nongenetically modified, since the cornea is a privileged organ; out of the immune systema action. The pancreatic islets re obtained from WT pigs because they are inserted in a protected way; as encapsulated islets. This strategy is not only used in xenotransplantation but also un allotransplantation, avoiding to transplant the exocrine portion of pancreas, that normally is working well in diabetics patients (Scharp & Marchetti, 2014). Briefly, the isolated pancreatic islets are surrounded by a capsule of different natures. The capsule allows a double-side diffusion; oxygen and nutrients needed by islets are internalized in the capsule and produced insulin is exchanged to the blood. Commonly these capsules are inserted in liver as a well irrigated organ, and because is not necessary to be the islets in the topographic area of pancreas. Usually, the capsules are not able to avoid the production of antibodies against the islets, but the encapsulated pig islet grafts have demonstrated to be efficacious in restoring long-term normoglycemia, suggesting that the coating was able to prevent contact of islet cells with both the host immune cells and antibodies

Current situation

Since 2021 the news about xenotransplantation arise to the front page of all media around the world. The reasons are two: the first kidney and heart xenotransplantation in a human preclinic model. The kidney xenotransplantation was announced on 20th October 2021, when a woman in brain death, not meeting donation conditions was donated to proceed with a kidney xenotransplant. The first reports were through media mass all over the world, and finally was scientifically communicated on December 2021 in the American Journal of Transplantation (Porret et al., 2021) showing the results of the first clinical-grade xenotransplant experience in vivo using a human decedent model. The pigs used were Revivicor polytransgenic pigs (GGTA1 KO/ β4GalNT2 KO/CMAH KO/ GHR KO/hCD55/hCD46/ hTBM/hEPCR/hCD47/hHO1), with 4 knockout genes and 6 human transgenes. The knockout looked for avoiding HAR and avoid the uncontrolled growth of the organ once implanted. The two complement complement-regulatory protein helped with this task and the TBM was introduced to overpass the coagulation problems observed formerly in animal model experiences. The CD47 and EPCR (Endothelial protein C receptor) genes were focused in controlling the inflammatory reaction. Both kidneys were transplanted separately using conventional heterotopic allotransplantation techniques. The right ureter was anastomosed to the decedent's bladder, and the left ureter was brought through the skin as an end urostomy. The survival of the kidney was 74 horas post-perfusion, but the endpoint was because logistic reasons and not due to a failure of the organ. Hyperacute rejection was not observed in this human decedent, providing critical evidence that knockout of the genes encoding enzymes that synthesize carbohydrate xenoantigens (i.e., GG TA1, β4GALNT2, CMAH) is indeed sufficient to prevent hyperacute rejection from this mechanism in humans. The most important is that this experience identified numerous areas where additional investigation is needed.

On January 10th, 2022, a xenotransplantation of hearth to Dave Bennet, a 57 years old alive man, was reported by mass media. The piece of news announced that a patient in terminal condition due to a heart failure, and assisted by an ECMO (Extracorporeal membrane of oxygenation) machine had been xenotransplanted with an Revivicor pig's heart. Two months of trying to save Bennett's own heart didn't work. A handful of transplant programs either formally or informally rejected him for a heart transplant. He was deemed ineligible for an artificial heart pump because of uncontrollable arrhythmia. Bennett didn't qualify for the list, because he had not followed doctors' orders, missed medical appointments and discontinued prescribed medications and the hope of the family was that after 6 months

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with a pig heart he will be able to follow the prescriptions; in fact, absolutely necessary for survive after a heart transplant. In the moment to write these lines (February 6th, 2022) there was no news of the health status of Bennet. The pig used in this experience was Revivicor's male of 120 Kg and 1 year-old with the same 10 genetic editions that in the former kidney xenotransplantation. In this case, there is not yet scientific communication of the findings.

Attitude towards xenotransplantation

One of the concerns of all the teams researching on xenotransplantation is the attitude of population towards this type of transplant. What is going to happened if after using a lot of resources and producing modified pigs, the people is not on favor to use animal organs with them? The research team at the University of Murcia has investigated the attitude of different population segments, by means of questionnaires. The opinion towards xenotransplantation, in general, use to be influenced by educational and economical levels and specially by the attitude towards all related to organ donation and allotransplantation attitude, but not by other psychosocial variables. Regarding the attitude towards human organ donation and xenotransplantation, the people on favor to donate their own organs is more in favor

towards receive a xenotransplantation. Other influencing factor use to be the closeness to the xenotransplantation experimentation; in fact, has been recorded important differences between hospital personnel in centers with and without research program on xenotransplant (Abalovich et al., 2017). Mitchell et al. (2019) in a metanalysis found as main factors influencing the attitude towards xenotransplantation (as those having a higher odds ratio) the personal experience with donation, the perceived benefit from a xenotransplantation, the partner be in favor of xenotransplant, the area of the country in which the participant lived, favorable attitude of one's religion toward xenotransplant, a favorable attitude toward cadaveric donation and whether or not one was a current organ donor.

In USA, a study of five focus groups, investigated the obstacles for xenotransplantation. Concerns were expressed primarily regarding issues of animal ethics, stigma regarding how pigs are viewed in society, organ allocation logistics, quality of life after receiving a xenograft, and how xenotransplantation would be accepted by certain theological traditions. But the consensus from the participants is that there is generally a high acceptance of xenotransplantation (Hurst et al., 2021).

Among the wide range of population segments, we have included in this text those studies related to the main actors in the equation: the patients in waiting list, and the students of veterinary, medicine and nursing students, since these professionals are important to generate opinion, and they will be involved in the xenotransplantation process in the future. And also, the secondary school students; since they are the population of the future that in medium-term will have to decide if they receive a xenotransplantation.

The patients in waiting list have been other population segment of interest in our studies, because they are direct candidates for a xenotransplantation in a direct way. In the case of kidney patients, if the results of xenotransplantation were as effective as those attained using human organs, 76% would be in favor, and in the case of liver patients, 67% would be in favor (Martinez-Alarcón et al., 2011). In other study in Type-1 diabetic insulin-dependent patients (whom could be considered as in waiting list for islets transplantation), they expressed that the requirements to accept an islets xenotransplant were an improved diabetes control and a reduction of diabetes-related burdens. Health-related aspects prove to be pivotal for diabetic patients when considering porcine islet cell transplantation, and the use of pigs as source for organ retrievals was not considered as problematic (Kögel et al., 2021).

Interestingly, the population most in favor to receive an animal organ in the case of similar results are the students of veterinary medicine, with 91% of veterinary degree Spanish students accepting xenotransplantation, 95% tissue and 97% cells (Martínez-Alarcón et al., 2010). And this attitude is not own of Spanish students, since we did not find differences among them and Brazilian veterinary sciences students; with a 90%, 94% and 97% of Brazilian accepting a xenotransplanted organ, tissue or cells, respectively (Mendonça et al., 2013). In both cases the attitude was not affected by the academic year, any psychosocial variable, or attitude toward organ donation.

Spanish medical students have a favorable attitude towards xenotransplantation. If the results of xenotransplantation were as good as in human donation, 81% of medical students would be in favor, 3% against and 16% undecided. The following variables affected this attitude: sex; academic year; discussion of transplantation with one's family and friends; the opinion of one's partner; the respondent's attitude towards organ donation; religion; and participation in altruistic activities (Ríos et al., 2015).

As regards nursing students, in a Spanish study involving 76% of students enrolled in nursing studies, if the results of xenotransplantation were as good as in human donation, 74% would be in favor and 22% would have doubts. The following variables affected this attitude: age; sex; geographical location; academic year of study; attitude toward organ donation; belief in the possibility of needing a transplant; discussion of transplantation with one's family and

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friends; and the opinion of one's partner (Martínez-Alarcón et al., 2019). This attitude was also observed in Poland (Mikla et al., 2015) were 62% of students were in favor of xenotransplant, being influencing factor, in this case, were the studies degree (more accepted by fourth degree students) and the religion (Catholics accept this type of donation more readily than those who belong to other faith traditions).

Among the students of secondary school in Spain 44% would be in favor, 22% against, and 34% undecided towards animal organ donation. Attitude was related to knowing a transplantation patient, believing that transplant organ needs are not covered, having received information about organ donation and transplantation on television and from schools, family discussion about organ donation, attitude of the respondent's parents, and attitude toward human donation (Febrero et al., 2018). In Belgium, a 36.1% of participants in a survey were in favor to xenotransplantation in the event of the same risks and result than in an allotransplant, 50% had doubts and 13.3% would refuse to accept an animal organ. It's interesting to observed than a 71.1% of the student had never heard about xenotransplantation. The prior knowledge would increase the acceptance up to 53% of the respondents (Reyneke et al., 2021).

From this information we have to learn important things: in general, the health care personnel that is going to be

directly involved in xenotransplantation in a near future (veterinarians, medicals and nurses) are very in favor towards xenotransplantation. These are good news since they have to build opinion in the patients and of course they have to agree working with animal's organs in the future. The best opinion in veterinary students could be explained by the direct relation with animals of this collective, meaning a more positive opinion of the animals.

The second important lesson is that the adults of the future (secondary school students) are less in favor that the health care professionals. This could mean that in the future the patients could have a no so positive attitude towards xenotransplantation. But the knowledge that the prior knowledge about the technique or that to receive information about donation in school or through television can improve the attitude show us the pathway. More effort has to be put in the education for the donation and xenotransplantation in the present and future. In fact, it would be a god proposal to include information about animal's organ donation in the general donation information campaigns. The future starts today, and of course is critical to educate the population. Trying to increase the donation rate and trying improve the perception of general population towards xenotransplantation.

Conclusions

I grew up in the world of xenotransplantation hearing a couple of sentences: one from Sir Roy Calne (1930 -), the surgeon doing the first liver transplant in Europe (1968), whom in 1995 said "Xenotransplantation is just around the corner, but it may be a very long corner" and the other from Norman Shamway (1923-2006), one of the pioneers in cardiac allotransplant: "Xenotransplantation is the future of transplantation and always will be". Apparently, we just are around the corner, and the future is coming really fast. The main reason: decades of research in animal model and the implementation of the newest genetic edition techniques. And all the history revolves around the reason for this congress: THE PIG. Once again, the pig is rendering an invaluable service to humanity, not only being one of the main protein sources but also being the ideal donor for xenotransplantation. But of course, it would be, as veterinarian, unjust to forget the hundreds of primates that have given their lives to be the graft in the research all over the world. Without them the pig-to-human xenotransplantation would need several more decades to become real.

Of course, is really naïve to think that xenotransplantation is going to solve the shortage of organs for transplant all over the world. The Spanish National Organization of Transplantation (ONT) estimates this year that only a 10% of the patients that potentially could survive or improves their quality of live receives an organ. This problem is going to be solved stimulating the human donation, opening new human organ sources as donation in asystole or living donation, but the pig is going to open the door to a new strategy: the bridge xenotransplantation, giving hope to the patient that is in fatal liver failure, and allow to the system to find the liver that needs in less than 24 hours. The liver of a modified pig would be the key to keep alive this patience whilst the human liver arrives. And now the corner seems to be shorter and shorter every day.

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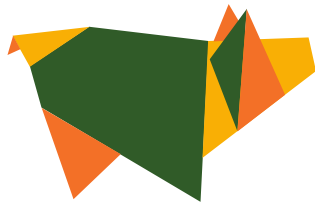
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