Application of the MISTEACHING(S) disease susceptibility framework to *Actinobacillus pleuropneumoniae* to identify research gaps: an exemplar of a veterinary pathogen

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Abstract

Historically, the MISTEACHING (microbiome, immunity, sex, temperature, environment, age, chance, history, inoculum, nutrition, genetics) framework has been applied to human pathogens. Here, we show, using *Actinobacillus pleuropneumoniae* as an exemplar, that the MISTEACHING framework can be applied to a strict veterinary pathogen, enabling the identification of major research gaps, the formulation of hypotheses whose study will lead to a greater understanding of pathogenic mechanisms, and/or improved prevention/therapeutic measures. We also suggest that the MISTEACHING framework should be extended with the inclusion of a ‘strain’ category, to become MISTEACHINGS. We conclude that the MISTEACHINGS framework can be applied to veterinary pathogens, whether they be bacteria, fungi, viruses, or parasites, and hope to stimulate others to use it to identify research gaps and to formulate hypotheses worthy of study with their own pathogens.

Introduction

One obvious question that arises in infectious disease, is why do some animals/humans become sick and/or die but others are resistant, survive illness or show no signs or symptoms of disease? This led Casadevall and Pirofski (2018) to a consideration of the factors that underly susceptibility to disease and the identification of 11 attributes that determine the outcome of host–microbe interaction. These factors were: Microbiome, Immunity, Sex, Temperature, Environment, Age, Chance, History, Inoculum, Nutrition, and Genetics, the first letters of which spell the acronym MISTEACHING. The MISTEACHING framework has been extensively applied to human (Casadevall and Pirofski, 2018) but not, to our knowledge, strict veterinary pathogens. The purpose of this review is to demonstrate the utility of the MISTEACHING framework by applying it to *Actinobacillus pleuropneumoniae* (APP), as an exemplar of a veterinary pathogen, to identify research gaps relevant to disease prevention and control.

Basic background to APP

APP is a bacterium that causes pleuropneumonia, a disease of pig lungs that causes substantial morbidity and mortality in the worldwide porcine industry (Bossé et al., 2002; Gottschalk and Broes, 2019; Gale and Valazquez, 2020). The dynamics of infection with APP were recently reviewed and are summarized in Fig. 1 (Sasso et al., 2018). Pigs, both domesticated and wild (Vengust et al., 2006), are the only natural host for APP, and the outcome of host–bacterial interaction is subclinical disease (no clinical signs, no lung lesions at slaughter), chronic disease (low mortality, few and/or low specific clinical signs, reduced growth rate and/or lung lesions at slaughter), acute disease (high or intermediate mortality, lung lesions at slaughter), tonsil colonization (which subsequently spreads to the lungs), or bacterial clearance (Gottschalk and Broes, 2019). APP is spread by direct contact and/or by aerosol (Tobias et al., 2013). Control is through a combination of husbandry, antibiotics, and vaccines (Gottschalk and Broes, 2019). However, the emergence of antimicrobial resistance is a major concern (Michael et al., 2018), and the most widely used bacterin (whole killed) vaccines only protect against one or, at best, a few of the 19 known serovars (Bossé et al., 2018c, 2018d; Stringer et al., 2021), and do not prevent colonization (Loera-Muro and Angulo, 2018). There is thus considered an urgent need for substantially improved and new prevention and therapeutic strategies.

Here, we show how application of the MISTEACHING framework to the veterinary pathogen APP can be used to identify gaps in our current knowledge, and to formulate hypotheses whose study will enhance prospects for disease control. It should be noted, as pointed out in...
the original MISTEACHING paper (Casadevall and Pirofski, 2018), that there is some overlap between the categories, and examples will be mentioned where appropriate. In addition, we propose, and present our reasoning for, an extra category – Strain – to be added to the MISTEACHING framework, so that it becomes MISTEACHINGS.

The MISTEACHINGS framework applied to APP

Microbiome

The two primary niches that APP occupies are the tonsils (Chiers et al., 1999) and the lung (Gottschalk and Broes, 2019), although recent data suggest that multi-organ spread of the bacterium, significantly correlated with spleen colonization, may occur during acute infection following lung colonization (Hoeltig et al., 2018). Studies of the tonsil microbiome of pigs that had no history of respiratory diseases and were considered free of APP, showed a post-birth early litter-related microbiome with high similarity to that of the sow teat skin and vagina. However, the tonsil microbiomes of individual litters converged over the following 3 weeks, and dramatically diverged at 4 weeks, with the stresses of a change in diet (weaning), change in room, and addition of in-feed antibiotic (Pena Cortes et al., 2018a). Notably, members of the family Pasteurellaceae, of which APP is a member, were present throughout the period, as they were in a longer study of 19 weeks (Pena Cortes et al., 2018b). The results were consistent with previous tonsil microbiome studies on 18–20 week healthy pigs (Lowe et al., 2011, 2012), and earlier studies reviewed by Kernaghan et al. (2012). APP can transmit from the sow to piglets as early as 10 days (Vigre et al., 2002). However, the effect of APP colonization on the tonsillar microbiome of healthy pigs is unknown. Not all litter mates will be colonized with APP, although the numbers increase with age (Tobias et al., 2014b), but whether there are bacterial species/consortia that can prevent tonsil colonization is also unknown, as is the effect of vaccination on the microbiome, and these can be considered as research gaps.

Similarly, there is a little information on the composition of the lung microbiome, either in health or disease. Siqueira et al. carried out a shotgun metagenomic study of lung lavage samples from a herd of pigs kept in field conditions with signs of infection with enzoonotic pneumonia, caused by Mycoplasma hyopneumoniae, or with no signs of infection (Siqueira et al., 2017). This descriptive study found that Mycoplasmataceae, Flavobacteriaceae, and Pasteurellaceae were the most common families identified in lungs with signs of enzoonotic pneumonia compared to Mycoplasmataceae, Bradyrhizobiaceae, and Flavobacteriaceae in those without signs of infection. A similar 16S rRNA-based microbiome study on lung lavages of slaughter pigs, with or without lung lesions, found microbial diversity was significantly reduced in lungs with severe-lesions compared to those with ‘slight-lesions’ or that were considered healthy (Huang et al., 2019). Mycoplasma and Ureaplasma were enriched in severe-lesion lungs, and 62 Operational Taxonomic Units (OTUs) were negatively associated with the presence of lung lesions, 49 of which were relatively low abundance (<0.05%). A similar 16S rRNA-based study of the lungs of healthy and diseased Duroc × Landrace × Yorkshire crossbreed pigs found a higher relative abundance of Lactococcus, Enterococcus, Staphylococcus, and Lactobacillus in healthy pig lungs and an enhanced richness of Streptococcus, Haemophilus, Pasteurella, and Bordetella genera in diseased lungs, although no diagnostics as to the primary or secondary infections of diseased pigs was reported (Li et al., 2020). Another 16S rRNA-based study comparing the diversity of bacteria in the alveolar lavage fluid obtained from healthy and diseased lungs of 4-week-old Kele pigs has also been reported (Zhang et al., 2020). Taken collectively, the results suggest that infection of pig lungs with bacterial pathogens can dramatically alter the microbiome, and that colonization with many divergent beneficial bacteria may have a role in protection of the lungs against pathogenic microorganisms. Whether APP infection of the lungs will also lead to a lack of bacterial diversity, and whether the presence of bacterial species/consortia can prevent infection is unknown. APP is a primary pathogen of the porcine respiratory disease complex (PRDC), where infection can involve multiple bacteria and/or viruses, so tonsil and lung microbiome-based studies may need to be monitored for polymicrobial infection, although the

![Fig. 1. Dynamics of infection with APP](https://www.cambridge.org/core) Adapted with permission from Sassu et al. (2018). See text for further details.
bacterium can be the primary agent of lung disease (Opriessnig et al., 2011; Saade et al., 2020), and initial experimental APP mono-infection studies may provide important baseline data. 

Also of interest is the interplay between the pig gut and lung microbiota (the gut–lung axis) and vice versa. Infection of specific pathogen free pigs with APP was associated with a change in the Simpson’s diversity index of fecal coliforms, indicating lower diversity in the gut (Zoric et al., 2010). Additionally, the richness score for taxa Ruminococcus_2 found in the guts of litters correlated significantly with the lowest lung lesions scores of animals experimentally infected with M. hyopneumoniae (Surendran Nair et al., 2019a). The results suggest that M. hyopneumoniae susceptibility is associated with early life gut microbiota. Similarly, the presence of non-pathogenic Escherichia coli and increased fecal microbiome diversity in the pig gut was associated with reduced lung pathology after experimental infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2, both agents of the PRDC (Niederwerder, 2017). Taken together, these studies suggest the possibility of targeted alteration of the pig gut microbiome, e.g. through probiotic or defined microbial consortia or purified metabolites (Wypych et al., 2019), to decrease susceptibility to lung disease caused by infectious agents, and future studies may also include inter-kingdom cross talk between bacteria, fungi and viruses which has been recognized as a component of healthy/diseased lung health (Enaud et al., 2020). Again, there is no information on whether the gut microbiome is associated with susceptibility to lung disease caused by APP, and the above discussion indicates there is a major gap in knowledge concerning APP and the pig tonsil/lung/gut microbiome.

Immunity

The immune status of an animal will clearly influence susceptibility to APP disease, and overlaps with many other categories in the MISTEACHING(S) framework e.g. Microbiome, Age, Environment, History, Nutrition, Genetics, and Strain. Historically, the main component considered in surviving an encounter with a pathogen – and therefore a component of infectious disease susceptibility – is disease resistance, i.e. the ability of the host to kill a pathogen (Ayres and Schneider, 2012). Immune-driven disease resistance mechanisms (including the innate, humoral, and cell-mediated responses) involved in elimination of APP have recently been reviewed in detail (Sassu et al., 2018) and will not be considered here. Examples demonstrating the importance of immunity, or lack of, to the outcome of APP-host interaction include: the substantial morbidity and mortality that arises when naïve animals, with/without preceding infection and/or co-infection with viruses, come into contact with asymptomatic carriers (Opriessnig et al., 2011), and that vaccination with bacterins can reduce severity of lung disease (Loera-Muro and Angulo, 2018). In general, such disease resistance mechanisms are poorly understood, and this can be considered a major research gap that needs addressing to formulate new prevention and/or treatment strategies.

In addition to disease resistance, it is now recognized that disease tolerance, i.e. the ability to maintain host fitness without affecting pathogen burden, is also an important determinant of host susceptibility to infectious disease (Soares et al., 2017). While the mechanisms that are involved in disease tolerance are not fully understood, they include tissue damage control mechanisms based on evolutionarily conserved stress and damage responses (Soares et al., 2017), and host metabolism and immune crosstalk (McCarville and Ayres, 2018). There can be considerable tissue damage during acute APP infection. Apx toxins and the induced inflammatory response can result in substantial lung damage, which can lead to chronic disease where characteristic abscess-like nodular lesions surrounded by connective tissue are a characteristic gross pathological finding (Gottschalk and Broes, 2019). Thus, tissue damage control mechanisms are apparent in the response to APP infection. Also associated with chronic disease is the persistence of APP in tonsillar crypts (Müllebner et al., 2018). Such animals maybe asymptomatic but are still capable of transmitting APP to naïve animals (Chiers et al., 2010). The host and bacterial mechanisms that allow APP to survive in the tonsillar crypts are poorly understood, and have significant control implications. A recent study analyzed cytokine responses in German Landrace pigs that had been infected intranasally with a serovar 2 strain of APP (Müllebner et al., 2018), and identified that acute disease was associated with increased expression of the pro-inflammatory interleukin-17A (IL-17A) in the lung, levels of which had previously been shown to correlate with the presence of lung lesions in chronically infected pigs (Sassu et al., 2017). It is known that Th17 immunopathology is largely driven by products of neutrophil activation, such as reactive oxygen species and elastase (Soares et al., 2017), and has parallels with work suggesting that Th17 responses are important in protection against human respiratory pathogens, e.g. Haemophilus influenzae (Noda et al., 2011), and Streptococcus pneumoniae (Ramos-Sevillano et al., 2019). In contrast, in APP-chronically infected tonsils there was increased expression of the anti-inflammatory cytokine IL-10. Indeed, in both lung and tonsil, there was a marked reciprocal correlation between IL-17A and IL-10 concentrations. IL-10 is considered a master regulator of immunity to infection, inhibiting macrophages, Th1 and natural killer cells, which can result in impedance of pathogen clearance at the expense of preventing excessive tissue damage (Couper et al., 2008). APP-infected pigs pre-treated with adenovirus-5 expressing human IL-10 had a significant reduction in lung damage compared to controls (Morrison et al., 2000). Müllebner et al. hypothesized that ‘APP adapts its metabolism to trigger IL-10 production and consequently facilitates chronic APP persistence inside porcine tonsillar tissue’ (Müllebner et al., 2018). From a bacterial perspective, the hypothesis is supported by differences found in Fourier transform infrared spectroscopy of serovar 2 isolates from lung and tonsillar tissue (Aper et al., 2020). That respiratory pathogens can induce IL-10 and control tissue damage, an example of diseases tolerance, is exemplified by Staphylococcus aureus (Chau et al., 2009). Further work is required to identify any APP factors involved in IL-10 induction and the host interactive biology, and such knowledge can potentially be used in formulating strategies to eliminate APP tonsillar carriage.

Also pertinent to disease tolerance is APP outbreaks associated with stress-inducing trigger factors in already colonized but asymptomatic animals (see Environment). Homeostasis (i.e. the ability of an entire organism, organ, or individual cell to maintain key regulated variables within an acceptable range) is a fundamental property of biological systems (Chovatiya and Medzhitov, 2014). Such parameters include oxygen, pH, glucose, and ATP. When these change beyond a certain threshold, host cells are alerted by stress sensors, such as pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular patterns, and induce signal transduction pathways that can restore
homeostasis. Receptors sensing general environmental stress (and infection) typically involve signaling through nuclear factor (NF)-κB, mitogen-activated protein kinases (MAPKs), and cJun NH2-terminal kinase (JNK) (Chovatiya and Medzhitov, 2014). In porcine alveolar macrophages, ApxI is known to induce apoptosis via MAPKs p38 and JNK (Wu et al., 2011), and IL-1β, IL-8, and TNF-α production via NF-κB in a JNK-dependent manner (Hsu et al., 2016). Metabolic adaptation to stress in host cells confers tissue damage control (Soares et al., 2017), in part by releasing metabolites, e.g. ATP, that influence innate immunity (Naquet et al., 2016) contributing to disease tolerance control (Soares et al., 2017). There is little information on tonsillar cell responses induced by stress triggers, but in transcriptomic studies of acute APP-infected lungs it was noteworthy that differentially expressed genes in necrotic areas were largely those involved in regulation of homeostasis (Mortensen et al., 2011). Understanding disease tolerance mechanisms in the context of APP colonization of tonsils has the potential for the formulation of therapeutic strategies that target stress and damage responses. Proof-of-principle that the general concept of targeting disease tolerance can work is shown by the prevention of tissue damage and lethality from sepsis following administration of the heme-sequestering protein, hemopexin, to mice (Larsen et al., 2016; Soares et al., 2017). Elimination of APP from the tonsils is notoriously difficult (Sassu et al., 2018), and currently complete restocking with APP-free animals is considered the only certain way of preventing the spread of infection. We believe that approaches to eliminate APP tonsillar colonization by immunomodulators targeting disease resistance (with or without antibiotics) is a worthy area of research. In addition, symbiotic microbiome-based approaches to reduce or prevent pathogen load (see Microbiome) could be considered as targeting disease tolerance, if they can be shown experimentally to induce immunoregulatory mechanisms and/or involve stress and damage responses (Soares et al., 2017).

Tissue damage control is also relevant to co-infections (Soares et al., 2017) and is considered a disease tolerance mechanism. Co-infection of an APP serovar 2 and an H1N1 influenza strain resulted in more severe symptoms and lung lesions and enhanced viral replication in the lung and nasal shedding, compared to pigs infected with single agents (Pomorska-Möl et al., 2017). The mechanisms by which particular viruses or bacteria compromise disease tolerance to secondary infections is likely to be multifactorial, with a degree of specificity for the combinations involved, but have been associated with T-regulatory (Treg) cell and amphiregulin dysregulation (Soares et al., 2017; McCarville and Ayres, 2018). Again, disease resistance and tolerance mechanisms preventing/limiting APP infection in the context of co-infections is a research gap with a need for more experimentally controlled studies, as undertaken by Pomorska-Möl (Pomorska-Möl et al., 2017).

The balance between disease resistance and tolerance will determine APP load and host damage (Fig. 2). However, this can be difficult to experimentally determine as: (1) the same cell types, e.g. macrophages, are involved in both disease

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**Fig. 2.** Schematic representation of the balance between disease tolerance (blue, LHS) and resistance (red, RHS) in relation to APP. Adapted with permission from Shourian and Qureshi (2019). TOP: During asymptomatic carriage there is a balance between host tolerance and resistance mechanisms, and infection is controlled. CENTRE: During acute disease initiated by stress events to asymptomatic carriers or acquisition of bacteria by naïve hosts, there is considerable damage to the lung caused directly by Apx toxins and also from the host. BOTTOM: Subsequently, APP is cleared from the lung and the host repairs the damage mediated by both the bacterium and host in fighting acute infection. At this stage APP can colonize the tonsils asymptptomatically (TOP), and the cycle continues. See text for further details.
resistance and tolerance; and (2) it often requires disease resistance to be stably maintained, e.g. through the use of antibiotics (Soares et al., 2017). One approach is to plot disease (health) parameters versus pathogen load over time, and the fitness curves can potentially be used to estimate variations in disease tolerance (Schneider, 2011). While APP load is relatively easy to quantify, choice of an informative health parameter to measure is considerably more difficult (Schneider, 2011). Nevertheless, such disease curves have the potential to identify parts of the APP disease process that are worthy of exploration. An alternative way of thinking, that reflects the overlap between disease tolerance and resistance, is to consider inflammation as the extreme end of a spectrum that ranges from hemostasis to stress response to the inflammatory response (Chovatiya and Medzhitov, 2014). In this model, inflammation can be triggered either by: (1) extreme deviation of regulated variables (e.g. temperature) from normal values, and is classed as a stress response; or (2) a challenge (e.g. infections, toxins, or tissue damage) that can cause deviation of regulated variables, but is not itself a regulated variable, and is classed as a defense response. Thus, inflammation has both stress and defense response components.

In summary, the basic immunology underlying APP-host interactive biology, and the contribution of disease tolerance to host control of APP, especially during chronic infection, can be considered as major research gaps.

Sex

That susceptibility to disease caused by a pathogen can be different between sexes (arising from anatomical, immune function, and genetic differences) is well established (Klein and Flanagan, 2013). While there has not been, to our knowledge, any description of APP metabolizing sex hormones, the closely related bacterium Aggregatibacter actinomyceseycomitans, which occupies the oral cavity of humans, can reduce testosterone to 5α-dihydrotestosterone (Soory, 1995). Microbiome composition can affect the response to vaccines (Ferreira et al., 2010), and is sex-dependent (Klein et al., 2015). This link has the potential for exploitation to maximize vaccine efficacy, which, for APP, may inform design of vaccines to prevent sow colonization.

Temperature

Fever (40.5–41 °C) is a clinical sign in acute disease cases due to APP (Gottschalk and Broes, 2019). Induction of fever, in response to infection and inflammatory disease, is a trait of warm- and cold-blooded vertebrates, conserved for over 600 million years (reviewed in Evans et al., 2015). The evidence suggests that, in general, a mild fever is considered of potential benefit but excessive fever maladaptive. The induction and maintenance of fever is known to involve a complex interaction of the innate immune system with neuronal circuits driven by the IL-6-COX2-PGE2 axis (Bernheim et al., 1979; Evans et al., 2015). The process is initiated after binding of PAMPs, e.g. LPS, to pathogen recognition receptors (PRRs), e.g. Toll-like receptor 4 (TLR4). In the case of APP, LPS-induced fever in rabbits (Maudsley et al., 1986), and endo-bronchial inoculation with ApxI and III, and to a lesser extent ApxII, induced fever in pigs (Kamp et al., 1997). Correlational antipyretic and hyperthermic/hypothermic studies suggest that fever is an adaptive response enhancing specific and non-specific immunity (Bernheim et al., 1979). For example, in experimental infection studies of New Zealand rabbits with Pasteurella multocida (like APP, a member of the Pasteurellaceae) there was a statistically significant correlation between fever magnitude and survival. An increase in fever of up to 2.25 °C was associated with an improved survival rate, but further increases with reduced survival rate. There was no difference in growth rate in vitro of P. multocida at normal (39 °C) and febrile (42 °C) temperatures, suggesting no direct inhibition of bacterial growth at increased temperature in rabbits, and the results were attributed to ‘an enhancement of some aspect of the rabbits’ immunological defences’ (Kluger and Vaughn, 1978). APP can grow at 42 °C for at least 8 h in vitro in rich medium supporting growth (Xie et al., 2013), thus it is likely (by analogy to P. multocida) that fever results in immune enhancement rather than direct killing during acute APP infection. Practically, detection of the rise in temperature associated with APP infection by thermal infrared imaging techniques has been evaluated as a diagnostic (Menzel et al., 2014; Jorquera-Chavez et al., 2020). As pointed out by Casadevall (2016), there are many studies that have correlated fever with survival, but none that unambiguously show causation. In part, this is due to the difficulty in determining how change in temperature affects both the immune system and the pathogen. Variation in temperature can also act as a stress trigger, increasing susceptibility to disease, and therefore overlaps with Environment and Immunity (see those sections for further details).

In the original MISTEACHING paper, temperature was considered from the host, but not bacterial, perspective. For many pathogens host body temperature is an environmental signal to induce the expression of virulence genes necessary for survival (Shapiro and Cowen, 2012; Lam et al., 2014). Typically, APP is grown in culture at 37 °C, but the bacterium can survive in the environment at room temperature for 3–4 days in the presence of mucin and salt, and on hydrophobic surfaces either under dry or saturated humidity conditions (Assavacheep and Rycroft, 2013). APP was cultured from farm drinking water in Mexico
and, in data not shown, it was reported that the bacterium could survive in water for at least 3 weeks at 20 °C in the laboratory (Loera-Muro et al., 2013). In addition, the authors detected the presence of APP in biofilm-like structures in the environment. It is not known whether APP found in water or environmental biofilms are as transmissible as those in aerosols or carried by fomites, and their genotype/phenotype (especially in respect to virulence factors) has not been evaluated. Such factors may influence indirect transmission and susceptibility to disease.

Environment

Environmental factors are well established as important determinants of susceptibility of pigs to respiratory disease – see the comprehensive review of Stark (2000). Risks factors associated with respiratory disease of pigs include purchase policy, herd type and size, husbandry system, stocking density, ventilation, air parameters (e.g. temperature), aerosol concentration, risks of contact and airborne infection, and hygiene (Stark, 2000; Neumann and Hall, 2019). Appropriate precautions and biosecurity measures should be put in place to reduce the risk of respiratory disease outbreaks. In particular, good herd management practices are necessary to reduce acute stress, e.g., from mixing, moving or weaning – so called ‘triggers’ of stress. Observational studies have indicated a link between changing environment and outbreaks of APP disease, suggesting an external trigger that results in an altered pig–APP interaction (Klinkenberg et al., 2014). Such a change could arise due to effects on pig homeostasis and/or the immune system (see Immunity), and/or the phenotype of the bacterium. Knowing whether clinical cases result from an external trigger in colonized pigs (trigger mechanism), or if an initial case precipitates an outbreak (transmission mechanism), is important in formulating optimal control strategies (Klinkenberg et al., 2014). A simulation using parameters derived from the literature suggested that APP outbreaks primarily arise from the trigger mechanism, with clear control implications, but that the relationship between subclinical lung lesions, antibodies, protection and disease warranted further study (Klinkenberg et al., 2014). Although one study (Maes et al., 2001) indicated that APP serovar may play a role in how risk factors (such as origins of purchased pigs and biosecurity) affect outcome of infection, it is noteworthy that there are few published studies directly addressing the effect of housing system on prevalence of APP disease. Temperature was considered as a risk factor for respiratory diseases in pigs bred outdoors (Beskow et al., 1998) and in units with natural, compared to mechanical, ventilation (Chantziaras et al., 2020). However, in general, respiratory disease is less prevalent in pigs reared outdoors compared to indoors (Delsart et al., 2020). Social and environmental enrichment had an impact on ‘disease susceptibility’ of pigs infected initially with PRRSV and 8 days later with a serovar 2 APP isolate (van Dixhoorn et al., 2016). Pigs in enriched conditions showed less stress behavior, and reduced disease susceptibility. In relation to APP, pigs raised in pens under enriched conditions had fewer lung lesions (7.1% of animals) compared to those raised in barren conditions (57% of animals). The authors suggested that the results supported the effect of housing on the hypothalamic–pituitary–adrenal (HPA) axis. Stress hormones epinephrine and nor-epinephrine are known to alter expression of APP genes, including those encoding virulence factors (Li et al., 2012), particularly those involved in regulation of growth, iron-acquisition and metabolism (Li et al., 2015), thereby linking with nutritional immunity (see Nutrition section). In summary, the evidence suggests that there is a clear link between environment and respiratory diseases in pigs, however this is an area where more controlled experiments would facilitate more robust correlations with disease caused by APP.

Age

Transmission from infected sows to piglets is known to occur from 10 days onwards, although not all litter mates, or all litters, are infected at the same time (Vigre et al., 2002). Suckling pigs rarely develop disease, especially if maternally derived antibodies provide protection. Maternal antibodies wane and are generally below detectable limits by 12 weeks of age (dependent on the test used), after which there is higher risk of disease (reviewed in Sassu et al., 2018). However, in naïve pigs, it has been reported that susceptibility to aerosol infection with APP was greater at 10 compared to 12 weeks of age, although no explanation was given for the results (Sebunya et al., 1983). Thus, in pig production systems worldwide, APP infection is predominantly a disease of pigs of 10 or more weeks of age. There are many unanswered questions relating to the immune mechanisms in sows versus those in piglets, especially relating to the nature and targets of maternal antibodies conferring protection against colonization. Understanding such mechanisms has clear implications for formulating sow and/or piglet-based strategies to prevent APP disease.

Chance

Many of the categories in the MISTEACHING framework of host susceptibility can be considered as stochastic, e.g. History, Inoculum, and Genetics (Casadevall and Pirofski, 2018). It could be argued that the element of chance of APP infection can be minimized in well run pig units, as the environment (e.g. temperature, ventilation, biosecurity, stocking density, etc.) can be tightly controlled. Transmission from pig to pig is by direct oral or nasal contact, or via aerosols over 1–2 m (Nicole et al., 1969; Kristensen et al., 2004), with direct contact being 10× more efficient than indirect transmission (Tobias et al., 2014a). There are, however, reported cases of indirect aerosol transmission between independent pig units (different organizations, no known contact between units) over distances of 500 m (Desrosiers and Moore, 1998; Larsen, 1998). Prior to one outbreak (case 2), weather reports indicated that the dominant winds were from the APP-infected farm to the one with no history of infection with the bacterium (Desrosiers and Moore, 1998). The strains involved were of the same serovar and antimicrobial sensitivity pattern. While identical serovar and antimicrobial sensitivity do not definitely prove the strains were the same, the data suggests the possibility of indirect transmission between unrelated units which, given the circumstances, could be considered as a chance event. With the advent of next generation sequencing technology and the application of single-nucleotide polymorphism (SNP) analyses, it is now possible to determine whether APP is transmissible over distances such as 500 m, as in case 2.

SNP analysis also facilitates detection of random genetic mutations, which – by their very nature – are chance occurrences, though environmental pressures selecting for persistence of the mutations can be controlled. In the host, random mutations can affect determinants of susceptibility and severity of infection, which through genetic mapping and selective breeding can be
used to improve resistance to specific diseases (see Immunity and Genetics). In bacteria, random mutations typically occur at frequencies between $10^{-7}$ and $10^{-10}$, but may be higher in the absence of functional mismatch repair (Lynch et al., 2016; Chevalleau et al., 2019). In addition to point mutations, spontaneous deletions or rearrangements in bacteria may be mediated by the presence of repeat elements in the chromosome that facilitate recombination, and horizontal gene transfer can lead to acquisition of new genes (Darmon and Leach, 2014). APP is a naturally transformable bacterium and therefore horizontal gene transfer can occur (Bosse et al., 2009). That chance events can alter phenotype is shown by inactivation of the expression of APP ApxIV (Tegetmeyer et al., 2008), as well as capsule and O-poly saccharide (To et al., 2020) of APP isolates, by insertion of ISAp1 into their cognate genes. Regardless of the mechanism, mutations which negatively affect fitness tend to be rapidly lost, whereas those increasing fitness become fixed in populations, giving rise to different lineages which may vary in virulence (see Strain, below). It should be noted that improved bacterial fitness does not necessarily equate to increased pathogenicity, as loss of virulence factors may be associated with increased persistence, due to reduced provocation of host immune responses. Part of the lung pathology in acute APP infection is due to release of toxic oxygen metabolites and inflammatory mediators by pulmonary macrophages activated by Apx toxin and LPS stimulation (Chen et al., 2011; Li et al., 2018), and deletion or insertional inactivation of these and other virulence genes by mobile genetic elements, such as ISAp1 (see above), are documented. Furthermore, in addition to loss or insertional inactivation of genes, acquisition of antimicrobial and/or other resistance genes by horizontal transfer (Michael et al., 2018) may also enhance persistence without increasing pathogenicity. In summary, chance events may influence susceptibility to disease, but these are typically difficult to determine.

**History**

There is clearly overlap between History and other categories in the MISTEACHING framework, particularly Microbiome, Immunity, and Environment. Heterologous immunity, i.e. infection with one microbe affecting the outcome of infection with a related one through changes in host immunological state, is a feature of APP. As a primary member of the PRDC, APP can itself alter the host immunological state, such that the pig is more or less susceptible to infection by other bacteria and viruses, and vice versa (Opriessnig et al., 2011). This phenomenon can be exploited for disease control. For example, a serovar 1 triple mutant of APP that expressed non-toxic but immunogenic ApxI, ApxII and ApxIV, induced significant protection in pigs against a serovar 5 isolate of Glaesserella (Haemophilus) parasuis (Fu et al., 2013). History is considered an important facet of control of APP. Many pig production units will choose to tolerate the presence of APP, providing that there are not serious outbreaks of acute disease. As such, APP is endemic in many countries, e.g. 98.2% of herds in Ireland were recently reported serologically positive for APP (Rodrigues da Costa et al., 2020).

The greatest risk of acute disease is from the introduction of asymptomatic carriers into a herd, exacerbated by the difficulty in identifying such animals (Gottschalk, 2015). Once in a herd, APP is difficult to eradicate. Options include: off-site segregated medicated early weaning supported by a program of vaccination; medication; culling and repopulation with disease-free gilts; on-site medicated early weaning; and 'test and removal' of sero-positive sows under medication (Gottschalk and Broes, 2019). Destocking and repopulation from herds which are certified free, or have no history, of APP infection is considered optimal. However, this is expensive and may lead to the loss of blood lines (Gottschalk and Broes, 2019), and once APP-free, strict bio-security is required to maintain the status. History also includes prior vaccination. The most widely used bacteria (whole cell killed) vaccines reduce the extent of lung lesions, do not prevent colonization, and only protect against homologous or closely related serovars. ApxI-III-based vaccines are aimed at neutralizing the effects of the various toxin combinations produced by different serovars, and there is considerable research in the area of live-attenuated vaccines, because of the potential to cross-protect against many serovars (reviewed in Loera-Muro and Angulo, 2018). Appropriate prior vaccination will reduce the susceptibility of pigs to APP-caused disease.

**Inoculum**

The effect of APP inoculum size in experimentally infected pigs has been the subject of many studies aimed at understanding host–APP interactive biology, and/or the efficacy testing of vaccines and therapeutics (Sassu et al., 2018). Both dose and route of administration can influence the outcome of infection. For example, pigs inoculated either intranasally (IN) or endotracheally (ET) with a serovar 1 clinical isolate resulted in infection (Baarsch et al., 2000). However, IN and ET inoculations were respectively associated with unilateral and bilateral gross lesions, and with clinical signs apparent between 6 and 8 h and <2 h. It was concluded that the ET route was superior for experimental infection, as all pigs were infected (unlike the IN route, where only 25% of animals had clinical signs 20 h post inoculation) and resulted in bilateral lesions characteristic of natural infections. With the same APP serovar 2 isolate, $10^5$ colony-forming units (CFU) and $4.9 \times 10^2$ CFU of intratracheal and IN doses, respectively, induced clinical signs and lung lesions, but not death (Henning-Pauka et al., 2008). Endobronchial inoculation with the reference serovar 9 strain CVJ13261, in doses ranging from $8 \times 10^5$ to $9 \times 10^4$ CFU, identified a unimodal relationship with the extent of clinical symptoms and severity of lesions. A dose of $10^4$ CFU was associated with the highest mortality and severest pneumatic lesions, while no death and less severe lesions were associated with a dose of $10^6$ CFU (van Leengoed and Kamp, 1989). Indirect transmission via aerosols is an established route of APP infection of pigs, and an aerosol dose relationship study in pigs with serovar 1 strain A79-9 found a correlation between inhaled dose and mortality, with LD50s of $7.0 \times 10^3$ and $1.9 \times 10^2$ CFU ml$^{-1}$ in the two separate experiments (Sebunya et al., 1983). In general, it was concluded that ET and endobronchial administration enable severe lung infection with a precise bacterial dose, but IN infection tends to lead to milder symptoms and chronic infection, in part due to the loss of the inoculum by coughing and swallowing (Sassu et al., 2018). In summary, APP infection is influenced by strain, dose and administration route.

**Nutrition**

That nutrition impacts immunity in pigs is well established (see excellent reviews of Liu et al., 2018; Pluske et al., 2018; Bouwens and Savelkoul, 2019). However, there has been little research done to predict the optimal diet for immune function.
in pigs, and such diets may differ from those that are required to avoid deficiencies (Chase and Lunney, 2019). That diet can affect the response of pigs to APP and other respiratory pathogens of pigs has been described (Turek et al., 1996; Becker et al., 2012; Surendran Nair et al., 2019a). For example, compared to control pigs fed an un-supplemented diet, those fed a diet supplemented with 5% garlic showed a lower incidence of lung lesions following aerosol-delivered APP serovar 2 (Becker et al., 2012). Experimental infection studies in pigs fed diets with different polyunsaturated fatty acids (PUFAs) found that there was a relationship between the content of \((n-3):(n-6)\) PUFAs in alveolar macrophages and outcome of \(M.\ hypopneumoniae\) infection (Turek et al., 1996). High-resolution liquid chromatography–mass spectrometry (LC–MS) analysis also found significantly increased serum \(\alpha\)-aminobutyric acid and long-chain fatty acids at 14 and 21 days post \(M.\ hypopneumoniae\) infection (Surendran Nair et al., 2019b). There is a lack of well-controlled studies regarding the effect of diet on the susceptibility of pigs to APP infection, especially those combining metabolomic analyses such as those by Surendran Nair et al. (2019b), which have the potential to rapidly advance our understanding and to improve control measures. Provision of feed for pigs is a major contributor to land and water use and greenhouse gas emissions, and sustainable environmentally friendly produced feed sources, such as insects, are under increasing investigation (DiGiacomo and Leury, 2019), and these have been associated with higher IgG blood levels compared to a conventional diet (Ko et al., 2020). This is an area which can be considered a major knowledge gap, and it is likely that future research will evaluate environmentally sustainably food approaches on the incidence of APP and other respiratory diseases in pigs.

Although not considered in the original MISTEACHING framework, nutritional immunity can be considered under this category, in addition to overlapping with Immunity. The phrase nutritional immunity was first introduced by Weinberg (1975), and refers to a host sequestering trace minerals, such as iron and zinc, to limit disease progression and severity after infection (reviewed in Hood and Skaar, 2012; Hennigar and McClung, 2016). Four days post aerosol infection with APP, there was an increase in total iron binding capacity, iron in serum, and zinc in plasma (Humann-Ziehank et al., 2014). APP can sequester iron from host transferrin, heme, hemoglobin, and haptoglobin through the ferric uptake regulator (Fur) protein-dependent expression of genes encoding transferrin binding proteins (Tbps), heme binding protein \(A\) (HbpA), hemoglobin binding protein (HgbA), and a possible hemoglobin–haptoglobin binding protein (HpuB), respectively (reviewed in Chiers et al., 2010). APP acquires zinc through genes encoded by the \(znu\)ABC operon (Yuan et al., 2014).

That iron and zinc are important for infection is clear from experimental studies in pigs. Firstly, surface-exposed APP iron and zinc-acquisition proteins are immunogenic (Goethe et al., 2000; Liao et al., 2009); secondly, attenuation of APP virulence in pigs can be achieved by mutation of genes involved in iron and zinc-acquisition, e.g. \(fur\) (Jacobsen et al., 2005) and \(znuA\) (Yuan et al., 2014). The data suggest that the ability of both host to sequester, and bacterium to obtain, iron and zinc contributes to disease susceptibility. That a prominent functional expression quantitative trait locus (eQTL) on \(Sus\ scrofa\) chromosome (SSC) 13, associated with resistance to APP infection, was found near the transferrin gene (Reiner et al., 2014b; see Genetics), and that among the six serovars investigated, the lowest expression of the three APP \(hp\)B ORFs was found in serovar 3 (Klitgaard et al., 2010), one of the lowest virulent serovars (Rosendal et al., 1985), also supports a role for nutritional immunity in APP disease susceptibility.

**Genetics**

In the original MISTEACHING framework, only host genetics was considered under this category, and again will only be considered here. We could also have included discussion of pathogen genetics under this heading, but believe that bacterial genomic and phenotypic differences are best included under a new category – ‘Strain’ – for the reasons given in that section (see below).

That some breeds of pigs are more likely to die from bacterial lung infection is well documented, and suggests host genetics contributes to susceptibility/resistance to respiratory diseases (Jones, 1969; Straw et al., 1983). Vaccines are widely used in the control of APP (Loera-Muro and Angulo, 2018), thus pig lines bred to have higher immune responses offer a route for enhanced protection, and this has been investigated (Magnunsson et al., 1997). A commercial whole-cell killed (bacterin) vaccine was administered to Yorkshire pigs selected for high immune response (HIR) or low immune response (LIR). It was concluded, based on the antibody response to carbohydrate and LPS antigens, that HIR pigs had a greater immune response to the bacterin vaccine than LIR pigs, and that ‘multi-trait selective breeding may be useful in facilitating vaccine-based health management programs for livestock.’ More recently, a genomic study of replacement gilts identified quantitative trait loci (QTLs) for antibody response to different serovars (1, 2, 3, 5, 7, 10, 12, and 13) of APP (Sanglard et al., 2020). Most QTLs identified were serovar-specific but one on SSC14 was associated with serovars 3, 5, 7 and 13, in a region associated with surface immunoglobulin IgM complexes. In total, genomic regions associated with the Ab response to the eight APP serovars were identified on 13 chromosomes. Several regions identified have been associated with reproductive traits in pigs, and the authors suggested that the QTLs could potentially be used for the improvement of resilience in commercial sows. That genetics is an important component of susceptibility/resistance to disease is also suggested by studies with transgenic pigs.

With a view to breeding pigs that are more resistant to APP, Hoelting et al. formulated a new respiratory health score (RHS) and tested the susceptibility of four pig breeds (i.e. German Landrace, Pïétrain, Hampshire, and Large White) to AP76, a serovar 7 strain of APP, delivered by aerosol (Hoelting et al., 2009). Based on the RHS, Hampshire and German Landrace pigs were the least and most susceptible to AP76 infection, respectively, confirming results from commercial farms (Jones, 1969; Straw et al., 1983). Follow-up studies to detect QTLs associated with susceptibility/resistance to AP76 on 170 Hampshire \times German Landrace F2 animals, identified significant QTLs on SSCs 2, 6, 12, 13, 16, 17 and 18 that explained 6–22% of phenotypic variance (Reiner et al., 2014a). One QTL on SSC2 reached significance on a genome-wide level for five associated phenotypic traits, and the genes \(IL-9\) (encoding interleukin 9), and \(CD14\) (encoding the cluster of differentiation 14 protein), known to be involved in the innate immune response to LPS, were considered candidates worthy of further investigation. A previous study by Gregersen et al. (2010) on 7470 pigs from crosses between 12 Danish Duroc boars and 604 sows (Danish Landrace \times Danish Large White) evaluated for dorsocaudal chronic pleuritis, which is a marker of pleuropneumonia (Gottschalk and Broes, 2019), had...
identified QTLs on SSC2, 8, 12, 13, 14 and 18 as being associated with resistance to APP. Thus, QTLs on SSC8 and SSC14 were detected only by Gregersen et al. (2010), SSCs 6, 16, and 17 by Reiner et al. (2014a), and SSCs 2, 12, 13, and 18 in both studies.

To prioritize candidate genes for fine mapping of gene variants, functional and transcriptomic analyses were carried out on the 50 most- and least-suscetible of the 170 pigs from the clinical QTL study (Reiner et al., 2014b). There were 171 differentially expressed genes between the two extremes of phenotype, and combined eQTL analyses (which identify associations between the expression of a specific gene and genotypes at different chromosomal locations) with network analyses and functional characterization, identified a functional hotspot on SSC13 which included 55 eQTLs. The most prominent of these 55 eQTLs, which explained 57% of total F2 variance, was a chromosomal region near the transferrin gene. APP can acquire iron for growth in the host by expression of Tbps (Gerlach et al., 1992), thus the result is biologically plausible. While many candidate genes were discovered, the authors concluded that ‘Further research will be needed to prove or reject their causal role in susceptibility to A. pleuropneumoniae’.

A further fine-mapping study used next generation sequencing to genotype 58 German Landrace pigs with the most extreme phenotypes after aerosol delivery of AP76, and a genome-wide association study (resistant versus susceptible to infection) identified SNPs on SSC2, SSC12 and SSC15, which combined explained 52.8% of the variance (Nietfeld et al., 2020). The significant variants on SSC2, which explained 32.9% of the phenotypic variance, were mostly intronic or intergenic, but some were in the gene encoding f-spondin (or SPONDIN-1). In mice, this gene is responsible for maintaining circadian rhythms (Carrillo et al., 2018), and is a negative regulator of bone mass (Palmer et al., 2014), whose cellular expression in vascular smooth muscle cells is increased by LPS binding. Specifically, LPS binds to TLR4 activating the PI3K/Akt signaling pathway, inducing f-spondin expression and subsequent proinflammatory IL-6 production, to promote vascular smooth muscle cell migration (Lee et al., 2016). IL-6 levels are known to dramatically increase during acute APP infection (Baarsch et al., 1995). The SNP on SSC12 is an intron variant of the platelet and endothelial cell adhesion molecule 1 gene (PECAM 1), and explained 19.9% of phenotypic variance (Nietfeld et al., 2020). PECAM-1 (also called CD31) is a glycoprotein that is a member of the immunoglobulin gene (Ig) superfamily (Lerkkiamongkol et al., 2016). In humans, PECAM-1 is expressed on the surface of granulocytes, monocytes, platelets, and endothelial cells where it functions to regulate vascular permeability and has a major role in leukocyte transendothelial migration (Privratsky et al., 2010). The facilitation of leukocyte transendothelial migration is considered pro-inflammatory, while functions including dampening of leukocyte activation, suppression of pro-inflammatory cytokine production, and maintenance of endothelial barrier integrity are considered anti-inflammatory. In pigs, PECAM-1 has been identified as the receptor for Clostridium perfringens beta-toxin (Bruggisser et al., 2020). PECAM-1 deficient mice were more sensitive to systemic administration of E. coli LPS than wild-type mice (Maas et al., 2005). LPS administration was associated with excessive accumulation of macrophages and neutrophils in the lungs of PECAM-1-deficient mice, and correlated with a prolonged increase in lung pro-inflammatory cytokine (e.g. IL-6 and monocyte chemoattractant protein-1), and chemokine C-X-C motif ligand 1) levels. The diminishing accumulation of cytokine-producing leukocytes, rather than regulation of cytokine synthesis by leukocytes was proposed to explain the results (Privratsky et al., 2010).

Infection with APP (or purified ApxI) results in apoptosis of porcine alveolar macrophages (Chien et al., 2009; Wang et al., 2015, 2016; Kim et al., 2019). Apoptotic cells are cleared by phagocytic cells involving ‘find me’ and ‘eat me’ signals (Grimsley and Ravichandran, 2003). PECAM-1 expression is known to be a ‘don’t eat-me’ signal protecting non-apoptotic cells from clearance (Brown et al., 2002). The above studies show that PECAM-1 has roles in inflammation and response to infection, and is a biologically plausible candidate worthy of further study in the context of APP infection. The variants on SSC15, associated with up to 18.5% of phenotypic variation, were in the COL4A4 region involved in the synthesis of type IV collagen, a backbone component of basement membranes. APP is known to bind to porcine lung-derived type IV (and types I, II, and III) collagen in vitro via an unidentified 60 kDa protein (Enriquez-Verdugo et al., 2004). It was speculated that the variants in the COL4A4 region on SSC15 may be part of mechanisms that restrict APP adhesion to pig lungs (Nietfeld et al., 2020).

Overexpression of porcine beta-defensin 2 (PBD-2) in transgenic pigs increased resistance (as adjudged by viable counts, lung severity scores, and histopathology) to APP intratracheal infection (Yang et al., 2015). The same pigs were also more resistant to G. parasuis (Huang et al., 2020). For some infectious diseases, the contribution of bacterial and host genetic factors to susceptibility has been assessed by calculating the sibling risk ratio, which measures the increased risk of disease in siblings of affected cases compared with the risk in the general population. For N. meningitidis, approximately one third of susceptibility to disease was attributed to host genetics, and two thirds to other predominantly bacterial factors (Haralambous et al., 2003). To our knowledge, no such analysis has been carried out with APP.

All of the above studies indicate that host genetic background is a contributor to resistance/susceptibility to APP infection, but that substantial further work is required to elucidate the identity of the specific genes/polymorphisms, and the underlying mechanisms involved, and whether such changes result in resistance/susceptibility to other pathogens as is the case with PBD-2 transgenic pigs. Also, before applying selective breeding, or even genome editing – which has recently been investigated for generation of pigs resistant to selected viral pathogens (Proudfoot et al., 2019), to alter specific genes for enhanced resistance to APP infection, it will be necessary to determine the effects of these mutations on other immune-related parameters (such as inflammation and stress) as well as overall health and production performance (Heuß et al., 2019; Ballester et al., 2020).

**Strain**

Here we propose that an additional category – Strain – be added to the MISTEACHING framework so that it becomes MISTEACHINGS. As originally formulated, the MISTEACHING model comprised only two categories that were clearly microbial-centric, i.e. Microbiome and Inoculum. APP has 19 serovars which are determined by surface carbohydrates, predominantly capsule (Bosse et al., 2018a; Stringer et al., 2021), and isolates express one or two of the ApxI-III toxins. ApxI is both strongly cytotoxic and haemolytic, ApxII is both weakly cytotoxic and hemolytic, and ApxIII is strongly cytotoxic (reviewed in Frey, 1995). In general, there is a correlation between serovar and...
ApxI-III expression, although exceptions are increasingly being found (reviewed in Gottschalk and Broes, 2019). Isolates expressing ApxI in combination with ApxII (serovars 1, 5, 9, 11 and 16) are considered to be of high virulence, and those expressing ApxII and ApxIII (serovars 2, 4, 6, 8 and 15) of medium virulence. Isolates expressing only one of Apx-I-III toxins are generally considered of low virulence (reviewed in Frey, 1995; Gottschalk and Broes, 2019). However, this is an oversimplification. For example, Jacobsen et al. found that serovar 2, 5, and 6 isolates were of equal virulence for pigs indicating that factors other than expression of ApxI and ApxII toxins are important (Jacobsen et al., 1996). While Apx-I-III toxins are unquestionably virulence factors involved in induction of lesions (Chiers et al., 2010), mutants producing wild-type Apx toxins, but with mutations in non-Apx related genes, can be attenuated (Bossé et al., 2002; Chiers et al., 2010). In particular, signature tagged mutagenesis studies in pigs led to the discovery of many attenuating mutations unrelated to expression of Apx toxins (Fuller et al., 2000; Sheehan et al., 2003). Encoded virulence factors were characterized as those involved in adhesion, acquisition of essential nutrients, avoiding host defense mechanisms, and persistence. An added complication is that the same mutation in different serovars may result in different phenotypic characteristics (Crispim et al., 2020). Compared to many other pig pathogens, e.g. Streptococcus suis (Weinert et al., 2015), there is a comparative lack of APP whole genome sequences available. Such availability would enable many more strain-specific questions to be addressed. For example, with the closely related human pathogen, H. influenzae, it was shown that a subset of genes was essential for survival in animals co-infected with influenza virus rather than the bacterium alone (Wong et al., 2013).

APP is a primary pathogen of the PRDC and co-infections with other bacteria and viruses (including influenza) occur. No information exists, to our knowledge, on whether specific strains of APP, through their gene content, have a survival advantage during co-infections with other microorganisms.

APP has been classified as intermediate clonal, based on multilocus enzyme electrophoresis (Messer et al., 1987; Møller et al., 1992) and amplified fragment-length polymorphism analysis (Kokotovic and Anjen, 2007). To our knowledge there is no conclusive published evidence of specific pathotypes of APP, e.g. clades associated with diseases or asymptomatic carriage but not associated with disease; even low virulence serovars, such as 3, can cause disease (Rosendal et al., 1985). This contrasts with G. parasuis, also a pig pathogen within the family Pasteurellaceae, which is highly diverse at the population level (Howell et al., 2014). In the case of G. parasuis, a pan-genome study identified 48 genes associated with clinical disease, of which a subset of 10 was used to formulate a pathotyping PCR to aid herd surveillance and disease control (Howell et al., 2017). Pathotypes have also been described for S. suis (Wileman et al., 2019), another bacterium that can cause disease in pigs. Similarly, for the human pathogen, N. meningitidis, some clonal complexes (such as CC11) are known to be disproportionately associated with invasive disease, while others have only been associated with carriage (Caugant and Brynildsrud, 2020).

Therefore, we suggest that the inoculum size is insufficiently descriptive of potential strain differences, especially where there is data suggesting pathotypes and/or specific clonal complexes being associated with disease. We, therefore, propose that the MISTEACHING framework be extended to MISTEACHINGS.
with the addition of the Strain category, and that a research gap with APP is the availability of whole genome sequences.

**Hypothesis generation and relation of MISTEACHINGS to other frameworks**

In their original publication, Casadevall and Pirofski (2018) indicated that the MISTEACHINGS framework was applicable to any pathogen, but the emphasis was on those affecting humans. Here we show that the framework can be applied to veterinary pathogens, using APP as an exemplar. We additionally show, based on the discussion in the 12 sections above, that the MISTEACHINGS framework can be used to formulate hypotheses worthy of study to aid disease control (Table 1). A recent review (Sassu et al., 2018) summarized gaps and challenges in APP research, identified under the DISease CONTROL TOOLS (DISCONTOOLS) framework (www.discontools.eu), but the recommendations made were practical and applied, e.g. improvements in vaccines, diagnostics, and treatment, rather than hypothesis generating. The MISTEACHINGS framework can be considered as less rigid than DISCONTOOLS (which also includes a scoring system), but both identified different areas of future research to control APP, and therefore the two frameworks can be considered as highly complementary. Both frameworks indicate that susceptibility of pigs to APP is a complex host–pathogen interaction, with all of the MISTEACHINGS categories (with the exception of Sex) contributing to disease susceptibility. We also propose, for the reasons given above, that the MISTEACHINGS framework be extended to MISTEACHINGS to take into account strain variation, especially to accommodate high levels of genetic diversity within a species.

**APP is a damage response framework (DRF) class 2 pathogen**

The original MISTEACHING framework was host-centric, with only Microbiome and Inoculum being microbial-centric. Thus, the addition of Strain extends the microbe-centric component of the framework (Fig. 3). While there are many definitions of microbiomes, most involve the microbiota, genomic content, and local environment (Casadevall and Pirofski, 2015; Berg et al., 2020), and it could be considered as both a host and microbial factor, although it is clearly predominantly microbial in nature. With Temperature, Chance, and Nutrition, we have also considered both microbe and host-centric standpoints. However, we consider that the host centric elements are stronger than the microbe-centric elements and, therefore, categorized them as host-centric (Fig. 3). It must be acknowledged that the contribution of Chance is difficult to assess, and likely to have a minimal contribution to APP disease susceptibility.

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with weak immune responses or in the setting of normal host responses. The rationale is that APP causes significant damage in naïve animals (weak host response) and those bacteria that survive acute infection induce antibodies (strong host response) but can nevertheless continue to colonize the tonsils (Fig. 4). It should be noted that in the context of the DRF, colonization is defined as ‘a state of host–microorganism interaction that leads to a variable amount of host damage, from minimal to great, thereby reflecting host immune responses that have the capacity to eliminate the microorganism.’ (Casadevall and Pirofski, 2003). APP also has two classic traits of Class 2 microorganisms, i.e. cause host damage by both pathogen (Apx toxins I-III) and host-mediated mechanisms, and that the immune response elicited by infection does not continue to damage the host once acute infection is resolved (Casadevall and Pirofski, 1999).

Conclusions

We have shown, using APP as an exemplar, that the MISTEACHINGS framework can be applied to a veterinary pathogen. We hope to stimulate others to use this framework to identify research gaps and to formulate hypotheses worthy of study in both veterinary research and teaching arenas with their veterinary pathogenic, whether they be bacteria, fungi, viruses, or parasites.

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