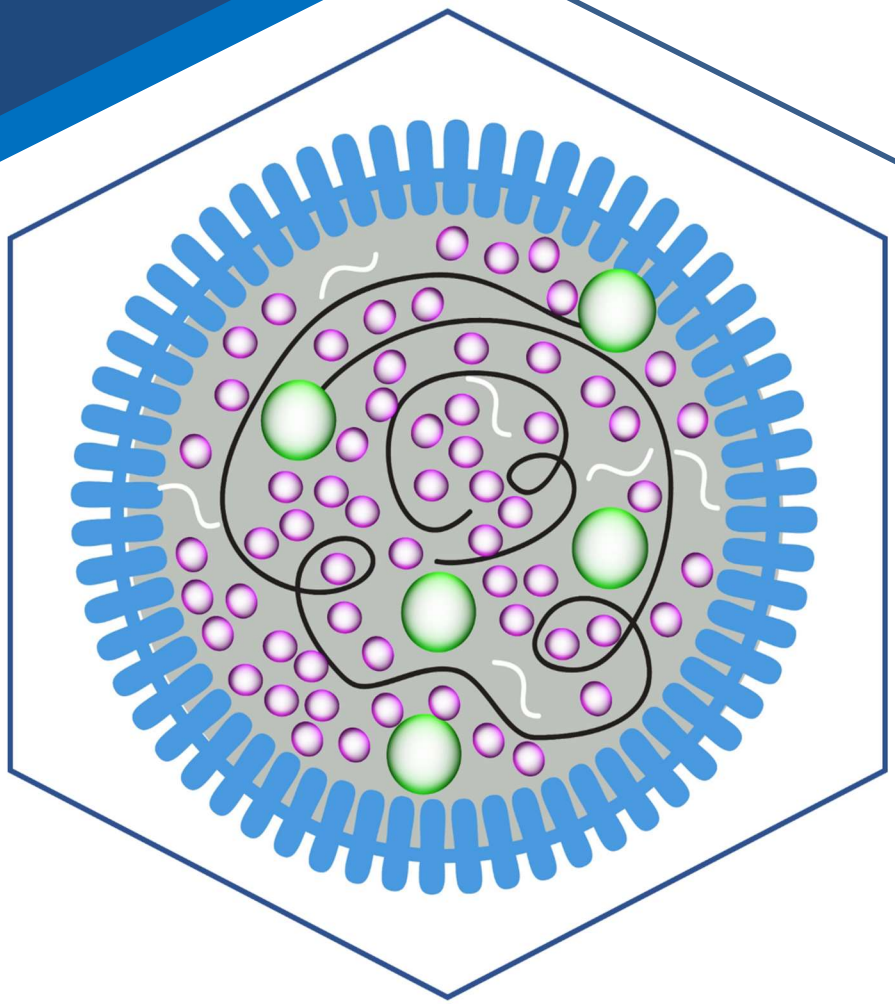
2023 Veterinary Mycoplasmas Research Report



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2023 Veterinary Mycoplasmas Research Report

Compiled, written & edited by

Drs Daniel Ackerman, Laura Roden & Lucy Robinson

Commissioned by

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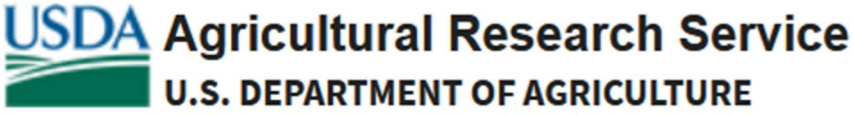

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Commissioning Body

This report was commissioned by the STAR-IDAZ International Research Consortium in collaboration with the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA).

The STAR-IDAZ International Research Consortium

The STAR-IDAZ International Research Consortium (IRC) is a global initiative aiming to coordinate research programmes at the international level and to contribute to the development of new and improved animal health strategies for priority diseases/infections/issues. The partners, research funders and programme owners together form the Executive Committee which is supported by the Scientific Committee of 16 experts and an EU-funded Secretariat (SIRCAH – Horizon Europe Grant Agreement Number 727494).

Target deliverables of the STAR-IDAZ IRC include candidate vaccines, diagnostics, therapeutics, other animal health products and procedures, and key scientific information/tools to support risk analysis and disease control. To achieve these goals, the IRC partners agree to coordinate/align their research programmes to address identified research needs relating to the priority topics and to share results. Research gaps identified by expert Working Groups are organised into research roadmaps for the development of candidate vaccines, diagnostics, therapeutics and disease control strategies, providing a structure to plot identified research gaps and focus future investment (Entrican et al., 2021).

The Agricultural Research Service, United States Department of Agriculture

The Agricultural Research Service (ARS) is the principal in-house research agency of the United States Department of Agriculture (USDA) and aims to extend the nation's scientific knowledge with research projects in agriculture, human nutrition, food safety, natural resources, and the environment. ARS supports more than 2,000 scientists organised into approximately 660 permanent research projects at over 90 locations across the country and five laboratories overseas.

ARS conducts innovative research to find solutions to problems of high national priority that impact the American people daily. ARS often undertakes high-risk research endeavours to make significant breakthroughs in important problem areas, including biodefence initiatives to detect, prevent, and mitigate the impact of especially dangerous infectious diseases that pose a threat to animals and public health.

Purpose of the Report

Mycoplasma species are responsible for several clinically important diseases of large ruminants, swine, and poultry, as well as affecting significant populations of related wildlife, some of which are becoming endangered or already under threat of extinction. Increasing our knowledge of these varied and challenging pathogens and their interactions with their equally diverse host species, is important for understanding the diseases they cause and so for enhancing our ability to control them with effective policies, therapeutics, and vaccines. This in turn is critical for food and economic security in many countries. These efforts should be focused on areas with the greatest potential for future advances, and this then requires regular updates of what is known and unknown in these fields of study.

The purpose of this report is to present and summarise some of the most important research published on these mycoplasmas over the past 8-10 years, covering their fundamental biology; diagnosis of infected herds and animals; pathogenesis and determinants of virulence; immunology and critical host-pathogen interactions; epidemiology and geographic spread; and control, including biosecurity, antimicrobials, and vaccine development.

The findings of this report are intended to be used to support future detailed gap analyses that will also incorporate expert opinion and review of current research and control measures, alongside knowledge of on-the-ground countermeasures (both in use and under development) and their efficacy. Importantly, this literature review does not attempt to rank the knowledge gaps identified, and this will therefore form a key part of future analyses.

About the Authors

Dr Daniel Ackerman

Dr Daniel Ackerman completed his Ph.D. in biological sciences and his subsequent post-doctoral studies at Carnegie Mellon University, Pittsburgh, USA. Since then, he has developed advanced communication and editing skills, joining Insight Editing London in 2020. Daniel has assisted with the publication of diverse research articles during his time with IEL, rapidly building his reputation for writing and editing excellence across fields. He also recently authored the STAR-IDAZ 2022 African Swine Fever Virus Research Review and co-authored the 2021 Animal Influenza Research Review.

Dr Laura Roden obtained her Ph.D. in Plant Sciences from the University of Cambridge, UK, and completed post-doctoral research at the University of Arizona, Tucson, Arizona and the University of Warwick, Coventry, UK, before establishing her own lab at the University of Cape Town, in South Africa. Alongside her academic work at Coventry University, UK, she holds an honorary post in the Department of Human Biology at the University of Cape Town. Laura joined Insight Editing London in 2022, bringing her talent for research review and grant writing to diverse fields and disciplines.

Dr Laura Roden

Dr Lucy Robinson

Dr Lucy Robinson gained her D.Phil. in veterinary immunology from the University of Oxford, UK, and continued her post-doctoral research into exotic infections at the Singapore Immunology Network. Lucy founded Insight Editing London in 2009 and has since successfully assisted with the publication of hundreds of articles and the construction of successful grant applications across diverse scientific fields. She recently directed the writing of, and co-authored, the Animal Influenza Research Review 2021, as well as editing the African Swine Fever Virus Research Review 2022.

Executive Summary

This report combines a comprehensive literature review with input from leading scientists across the field (for details of contributors, see here) to describe the significant progress made in research into mycoplasmas affecting veterinary species of interest, between 1st January 2012/2015 and May 10th 2023. Herein we provide a literature-based update and summary of notable research, that allows for the identification of some of the areas in which future research and research funding could be targeted for maximum impact and minimum duplication of effort. The priority research areas presented within this report are intended to be used as a tool to supplement future in-depth gap analyses that include additional factors and local/regional considerations.

Despite substantial research efforts and notable advances in knowledge, veterinary mycoplasmas continue to cause a suite of diseases that represents a major global threat to food security and animal welfare; this is most keenly felt by small producers in low-resource settings, who have a real and immediate sense of economic loss and food (in)security upon the onset of illness within their herd. While the mycoplasmas discussed within this report, and their corresponding host species, are diverse, common themes emerge from the literature review that warrant attention across the field:

- the need for standardisation of challenge models for *in vivo* pathogenesis, immunology and vaccine efficacy studies
- the value of genome sequencing, when coupled with analysis of the proteome, secretome and metabolome, to link genetic traits with *in vivo* pathogenesis
- the importance of inexpensive and accurate pen-side (requiring minimal laboratory equipment) diagnostics as the foundation of effective surveillance, epidemiology, and control programmes
- communication with primary stakeholders to ensure translation of research findings into effective practices in the field
- the risks of *in vitro* research in cell lines not mimicking the responses to infection in primary cells or *in vivo*
- the need to phase out immunological/pathogenesis studies of mycoplasmas affecting large animals conducted in inappropriate small animal models; for example, the data so far do not indicate that rabbits can model the immune recognition of pigs, nor that mice can replicate the responses to mycoplasma of cattle, and we therefore cannot rely on data generated in these systems

- exploitation of advances in technology and tissue culture – e.g., bovine lung organoids, and "lung-on-chip" approaches – could prove productive across the field
- similarly, the use of next-generation immunological techniques, such as single-cell RNA-seq, would give an unprecedented level of insight and generate data on which to base targeted studies with the potential to rapidly advance knowledge
- high-density, high-animal-stress farming and transport practices significantly lower the disease resistance of animals and may adversely affect their responses to vaccines, thereby favouring disease-transmission. The move to more sustainable farming practices is not only environmentally indicated but could also significantly reduce the animal disease burden in modern agriculture.

Alongside these repeating themes, a summary of *Mycoplasma* species-specific findings is reported below.

Mycoplasma mycoides subsp. mycoides (Mmm)

The continuing spread of contagious bovine pleuropneumonia (CBPP) represents a real and immediate threat to livestock keepers in sub-Saharan Africa, alongside the risk of the disease spreading to other countries. The worsening of the situation reflects a lack of effective and coordinated disease surveillance, containment, and mitigation strategies, which in turn is underpinned by an incomplete knowledge of the basic biology, pathogenesis, and immunology of *Mmm*. Since 2012, research into these areas has begun to present new opportunities for improved diagnostics, vaccines, and other mitigation measures, with the prospect of better control of CBPP within endemic regions. However, the translation of these findings into real-world benefits will require prioritisation of CBPP by national governments and international bodies: the potential gains from effective CBPP control are huge, not only to individual farmers within these regions, but also to global food security and for increased international trade, while the consequences of continued neglect of this disease are similarly profound.

Epidemiological data show that CBPP continues to spread within sub-Saharan Africa, with some reports suggesting that *Mmm* may also be circulating in parts of southeast Asia, as well as in Pakistan (and likely, northern India). The situation is almost certainly worse than these reports suggest, given the lack of access to diagnostic tools and the absence of comprehensive surveillance strategies in many regions. At present, the diagnosis of CBPP tends to be achieved at the herd level, but advances

are being made: both highly-sensitive loop-mediated isothermal amplification (LAMP) assay kits and ELISA-based lateral-flow devices have been tested and represent a promising way forwards for specific and rapid point-of-care diagnosis.

Efforts to advance understanding of the biology of *Mmm* have been notably advanced by genome sequencing, which has begun to reveal virulence factors of this species that might later be targeted in attempts at rational attenuation to generate improved vaccine strains. Further proteomic study of the pathogen *in vitro,* ideally using the improved primary cell/tissue culture methods reported herein, will advance this field further. In the living host, our understanding of the interactions between *Mmm* and the immune system is far from complete: a general consensus has emerged on the importance of TNF-α and neutrophils in the initial stages of infection, but we do not yet understand how antibodies act to either enhance or reduce symptoms of disease. Moreover, T cells seem important for the formation of immunological memory yet completely dispensable for protection from acute infection, and many questions remain as to how these data fit together. Across *in vivo* studies of immunology and pathology, the success of collaborations between field-study centres in affected regions, and distant laboratories with additional expertise and analytical capabilities, stands out: in the absence of funding to equip local research institutes to carry out high-impact cutting-edge research, this approach should be encouraged. Alongside, solid data reveal the importance of moving away from intra-tracheal challenge for all large-animal studies of CBPP: this should be applied as a matter of urgency, at the risk of rendering future research less-than-valuable when generated in this system.

The need for a standardised challenge method reflects a broader need within the field for standardisation of research approaches, most notably for vaccine research, to enable comparison of studies and the accumulation of knowledge. Current vaccines do somewhat reduce average disease severity and can be used as part of effective control programmes; yet the situation “on the ground” remains dire, with modelling data seeming to suggest that, given the shortfalls in their efficacy, immunisation with the current vaccines is worse than not vaccinating at all. While research continues towards an ideal vaccine, given the dearth of funding and the urgency of the situation, the most rapid path to improved control might well be best supported by prioritising the further testing, optimisation, and commercialisation of existing lead candidates. A vaccine that was even slightly more effective than current options, but better tolerated, and – importantly – more easily, cheaply, and reliably produced, would represent a major step forward for CBPP control.

An improved vaccine would need to be applied as part of a similarly improved vaccination campaign, aiming for at least 80% coverage. The prevalence and relative severity of post-vaccinal reactions to commercial CBPP vaccines has made the development of current and future vaccination programmes extremely challenging: farmers simply do not want to vaccinate their animals, and compliance is accordingly low. A worrying sequela is increased use of antimicrobial drugs, which is a global concern in terms of the emergence of resistant strains and possible masking of symptoms, allowing for further spread of disease. Therefore, recent research into the factors affecting farmers' decision-making around CBPP diagnosis and control has generated important insight that should be placed at the heart of future policymaking around this disease to ensure that future efforts to control CBPP achieve greater success than those of the past.

Mycoplasma bovis

Mycoplasma bovis (*M. bovis*) is one of the most pathogenic members of the *Mycoplasma* genus and pervades cattle farming globally, as well as infecting wild American bison and pronghorn. Since 2012, *M. bovis* has colonised Finland, which was previously a rare disease-free stronghold, reflecting its overall apparently increasing prevalence around the world. Alongside this organism's role in the bovine respiratory disease (BRD) complex, *M. bovis* also commonly causes mastitis and arthritis, which are poorly responsive to antimicrobial drug treatment. In the absence of an effective vaccine, most countries attempt to limit the spread of the pathogen by slaughtering all infected animals; though recent studies suggest that an infected case isolation approach could be as good or better, this has yet to be assessed in the field.

Since 2012, molecular typing has begun to shed light on the evolution and population structure of *M. bovis* isolates from the field, as well as identifying genomic factors involved in transmission. Complete genome sequencing has also advanced our knowledge of strain-specific pathogenic factors, and is now affordable enough to be used for contact tracing and identification of the source of outbreaks in developed countries; while the relative scarcity of material and human resources, and access to specialised laboratory space, preclude the translation of this benefit to low- and middle-income countries.

While some hoped that genome sequencing would have also opened the door to prediction of the phenotype and tissue specificity of different *M. bovis* isolates, this is not yet the case; similarly, genomic/molecular/pathogenicity studies have yet to reveal the basis for the transition from

asymptomatic colonisation to pathogenic infection, as often seen in respiratory infections with *M. bovis*, leading some to suggest that it is (yet undefined) host- or co-infection- derived factors that drive this process. It has, however, become clear that there are important differences in genome and pathogenicity between isolates infecting cattle and those from bison. Newfound abilities to generate targeted *M. bovis* mutants should facilitate a more thorough understanding of these processes and differences in the coming years and help to close the gap between our knowledge of the genetics, proteomics and *in vivo* biology of this pathogen.

The quest for accurate, affordable, and easy-to-use *M. bovis* diagnostics has progressed significantly since 2012. Multiplex PCR assays have now achieved 100% specificity and more than 90% sensitivity, representing a good option for those working in high-resource settings, while LAMP-based protocols may also be accurate and are somewhat more affordable to use. Although historically less sensitive than DNA-based approaches, recent advances hint at the prospect of highly sensitive serology-based lateral-flow devices that show promise under laboratory conditions but require further testing in the field.

Many of the recent studies into the pathogenesis and molecular immunology of *M. bovis* infection have been conducted *in vitro* using immortalised cell lines, or in some cases primary or *ex vivo*-derived cells. Much work lies ahead to validate their potential significance *in vivo* in order to identify those candidates that might be amenable to the generation of rationally attenuated mutants and/or as novel drug targets. Meanwhile, advances in our knowledge of the immune response to *M. bovis* infection of cattle have suggested key features of a protective adaptive immune response that could be targeted in vaccine design: several studies together provide evidence that Th17/Th2 is a hallmark of more severe and/or persistent infection with *M. bovis*, while features of Th1 immunity appear to be linked to protection (at least following vaccination).

Whether or not individual animal's varying susceptibility to *M. bovis/*BRD relates to T cell polarisation, or rather to innate immune traits, remains to be seen. Little work has been done in this area, yet interesting data suggest that such work could be highly productive: for example, the discovery that innate immune loci could be involved in resistance to *M. bovis*, coupled with advances in our knowledge of potentially important molecules such as the NK lysins, which show clear anti-mycoplasma activity *in vitro*.

Alongside the potential for breeding in increased *M. bovis/*BRD resistance, the first live-attenuated commercial vaccine against *M. bovis* "Protivity™" was launched by Zoetis in 2022, and offers hope of improved control, if its field efficacy aligns with that documented under controlled conditions. One potential issue is how such a vaccine might interfere with diagnostic testing; therefore, a more promising approach might be the further development of the reported attenuated *Mannheimia haemolytica*-vectored *M. bovis* vaccine expressing the cross-strain-conserved Elongation factor Tu and heat-shock protein-70. At the same time, preliminary data suggest that plant-derived antimicrobial agents, such as carvacrol, could also help in the fight against *M. bovis* disease in the near future.

Other mycoplasmas affecting cattle

Cattle are one of the most important livestock animals worldwide and are a keystone of food security and socioeconomic development in many countries. Pathogens that impact their health and productivity are therefore critical targets for veterinary researchers, and advances in our understanding of and ability to control cattle diseases can have an outsized effect on global welfare if put into practice. Numerous mycoplasmas are considered pathogens of cattle and are regularly isolated from cattle tissues, but our knowledge of their biology and prevalence is generally quite limited. As mycoplasmas regularly occur in co-infections with other mycoplasmas, other bacterial species, or viral pathogens, distinguishing the contributions of these individual pathogens to disease can be exceedingly difficult.

Mycoplasma bovigenitalium is often detected in bovine genital regions and is usually associated with reproductive disorders, but it has also been detected in the respiratory tract of diseased cows. The pathogenicity of *M. bovigenitalium* is thought to vary from strain-to-strain, and few in-depth studies have been conducted on its molecular properties or epidemiology. The past eight years have seen the publication of new genome sequences from *M. bovigenitalium* isolates and the development of multiplex diagnostics that can distinguish this bacterium from other commonly isolated pathogens (e.g., *M. bovis* in respiratory swabs and *Ureaplasma diversum* in vaginal swabs). However, many questions about the mechanisms by which *M. bovigenitalium* may (or may not) cause reproductive and/or respiratory dysfunction remain to be answered.

While *M. bovis* dominates the research field of bovine respiratory mycoplasmas, more minor players including *M. dispar* and *M. bovirhinis* have been addressed in a number of studies released since 2015. Several genome sequences and some accompanying annotation studies have been published, and the

prevalence of *M. dispar* has been reported in surveys of bovine respiratory disease (BRD) complex-associated pathogens in Europe. The sensitivity of *M. dispar* isolates to specific antimicrobials has also been studied, which is particularly important as the prevalence of drug-resistant mycoplasmas continues to rise.

The third class of bovine mycoplasmas addressed in this section is the haemotropic mycoplasmas (haemoplasmas), primarily *M. wenyonii*. *M. wenyonii* has been associated with severe anaemia in cattle, and the likely role of arthropod vectors in spreading this pathogen raise additional uncertainties in its epidemiology. The bovine haemoplasmas have seen increasing research attention since 2015, including the development of new diagnostic assays for their detection in blood samples. The prevalence of these pathogens has been investigated in countries across the world and has generally been found to be worryingly high. As our knowledge of the basic biology and pathogenicity of bovine haemoplasmas continues to increase, it will be particularly important to apply these findings to the establishment of new surveillance programmes to ensure that the spread of haemoplasmas can be tracked and, eventually, controlled.

Mycoplasmas affecting swine

Mycoplasma infections of the swine respiratory tract reduce welfare and productivity in domestic pigs across the world, and limitations in our ability to monitor and control these diseases introduce substantial uncertainty into pig farming networks. The most prominent pathogenic mycoplasma affecting swine if *Mycoplasma hyopneumoniae* (*Mhp*), the primary causative agent of swine enzootic pneumonia. Infection with *Mhp* alone has been associated with lost productivity (e.g., lower average daily weight gain), but the primary threat from this pathogen comes from co-infections – *Mhp* is very commonly observed alongside other major swine respiratory pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza A virus (sIAV), with complicated synergistic effects that can aggravate the severity of clinical signs in co-infected animals.

The eight years have seen numerous advances in our understanding of the biology of *Mhp* and its interactions with host cells. Multilocus molecular typing strategies based on just three genes (*adk*, *rpoB* and *tpiA*) have been found to provide high-resolution discrimination between even closely related strains of *Mhp*, simplifying phylogenetic analyses and facilitating a large number of studies into the evolutionary history and transmission networks of this pathogen. *In vitro* and *ex vivo* studies of the *Mhp* proteome and its interactions with host proteins have identified new putative virulence

factors and immune evasion-associated proteins that appear to play critical roles in the *Mhp* infection pathway and represent promising targets for the development of new therapeutics and vaccines. Some of these results have been validated *in vivo*, but many findings remain to be tested in experimentally infected animals. Standardisation of these *in vivo* studies will be an important goal: as the route of inoculation is likely to affect pathogenesis and immunity, physiological challenge models should be developed (where lacking), validated and standardised for use in studies of pathogenesis, immunology and vaccine efficacy. Similarly, the use of immortalised cell lines should be discouraged, as should the use of rabbits for vaccine trials: evidence is emerging that they recognise different epitopes.

Meanwhile, studies of swine enzootic pneumonia management and control have continued apace. Many studies have been published on *Mhp* prevalence in endemic areas of Asia, Europe, and the Americas, though very few of these studies have focused on Africa, and *Mhp* epidemiology within this continent remains an important research gap. The risk factors associated with *Mhp* introduction and transmission within domestic pig herds and wild boar populations have been investigated in a variety of geographical settings. Alongside, new diagnostics have been developed, including multiplex assays for discriminating *Mhp* from other swine respiratory pathogens and isothermal assays for improved pen-side performance.

New serodiagnostics for *Mhp* have been reported to discriminate infected from vaccinated animals, which is particularly important as our repertoire of vaccines against *Mhp* continues to grow. Fostera gold, the new trivalent *Mhp* vaccine, seems highly effective under a range of field conditions, yet the potential benefits of long-term use have yet to be revealed. If and when other novel vaccines are developed, it will be important to compare these vaccines against Fostera gold to ensure the selection of optimal vaccine strains in the field. Meanwhile, needle-free vaccination does not impact the efficacy of the commercially available vaccines and should therefore be more widely adopted for reasons of animal welfare, administrator safety and reduction in the substantial issue of sharps waste generation from veterinary vaccination programmes.

Alongside this progress, many advances have recently been reported in the study of other swine respiratory mycoplasmas – namely *M. flocculare* (*Mfl*), *M. hyorhinis* (*Mhr*), and *M. hyosynoviae* (*Mhs*) – and of the pathogenic swine haemoplasmas – *Mycoplasma suis* (*M. suis*) and the newly identified *Candidatus* Mycoplasma haemosuis (*C.* M. haemosuis). Studies of the proteomes and secretomes of these less-pathogenic respiratory mycoplasmas have identified some of the likely determinants of the

comparatively greater virulence seen in *Mhp*, and surveillance programmes have begun to generate critical data on the poorly understood epidemiology of swine haemoplasmas (at least, in Europe and the Americas). Further research will be needed to expand our knowledge of the haemoplasmas in Asia and Africa.

Mycoplasmas affecting poultry

The most important pathogenic mycoplasmas of poultry, *Mycoplasma gallisepticum* (*M. gal*) and *Mycoplasma synoviae* (*M. syn*), cause acute or chronic respiratory disease and associated productivity loss in domestic chickens and turkeys worldwide. Domestic chickens are by far the most numerous livestock animal on the planet, and mycoplasmal infections place a proportionately large economic burden on livestock production networks. As with veterinary mycoplasmas in other livestock species, *M. gal* and *M. syn* infections often occur in combination with other bacterial and viral pathogens of poultry, potentially increasing pathogenicity and negatively impacting the host immune response. This combined with the highly transmissible nature of these pathogens promotes complicated and difficult-to-predict epidemiological patterns, but the current lack of sufficient disease surveillance and control programmes in many countries limits the resources available for pathogen tracking, outbreak prediction, and the establishment of biosecurity measures across much of the globe. In light of these critical needs, this section of the research review will cover the many important strides that have been made over the past decade in clarifying the biology, epidemiology and control of *M. gal* and *M. syn*.

M. gal has been considered a major poultry pathogen for decades, and papers published since 2015 have added substantially to a large existing repertoire of studies. Advances have been reported in the typing and classification of *M. gal* isolates to support phylogenetic, evolutionary and epidemiological studies, while large- and small-scale functional studies of this pathogen's proteome have identified new putative virulence factors involved in cell adhesion, leucocyte chemoattraction, host cell apoptosis and other critical host-cell interactions. *In vitro* models have allowed detailed characterisation of the many proinflammatory responses triggered by *M. gal* infection (e.g., in innate immune signalling pathways, proinflammatory microRNA production and cytokine regulation), though many of these findings remain to be validated *in vivo*. Alongside, new developments in diagnostics are facilitating a slow but apparently steady increase in the coverage of surveillance studies, though the transmission patterns of *M. gal* remain poorly understood in many endemic areas (particularly in low- and middle-income countries). Testing of wild birds for *M. gal* is also generally limited, though many

studies have been conducted over the past eight years in the house finch (*Haemorhous mexicanus*) populations of the USA.

Meanwhile, although numerous commercial vaccines against *M. gal* have been available for decades, but their efficacy in controlling disease is limited by inconsistent protective efficacy and the frequency of chronic *M. gal* infections. Studies published since 2015 have begun to address these issues by further characterising existing vaccines and/or developing new vaccines with improved clinical properties.

Many of the limitations on our knowledge of *M. gal* (many uncharacterised gene products, insufficient surveillance in endemic areas, etc.) apply also to *M. syn*. *M. syn* was previously considered of less clinical importance compared to *M. gal*, and our research knowledgebase concerning this pathogen was correspondingly limited. However, an increase over the past 15 years in *M. syn*-associated synovitis and weakened eggshells in infected poultry has spurred a massive increase in the number of papers addressing it. The development of multilocus molecular typing protocols has allowed higher resolution phylogenetic characterisation than was previously possible with traditional *vlhA* gene sequencing, and studies of the host transcriptional and proteomic responses to *M. syn* infection have begun to clarify the molecular mechanisms of synovitis and immune dysregulation associated with this pathogen – though the host immune response to *M. syn* and its corresponding immune evasion strategies remain poorly understood. Meanwhile, the prevalence of *M. syn* among domestic poultry has been reported for the first time in some countries, and studies of its transmission have identified vertical transmission as a potentially important factor in *M. syn* epidemiology. *M. syn* has also been the focus of several recent studies on antimicrobial susceptibility, critical for tracking the development of drug resistance.

Literature Review and Research Updates by Subject Area

Report approach

The primary literature review was conducted using the applied life sciences CAB Abstracts database (www.cabdirect.org) with search terms specific to the species, pathogens, and date range of interest, starting on 1st January of the earliest year, until 8th May of 2023. For a full list of the search terms used, please see here. This approach yielded a total of 2,829 papers across the species of interest. Papers were subsequently excluded from further consideration if they were not published in English or not relevant to the topic. The remaining papers were then manually screened for relevance to the scope of this report, finally leaving 1,773 papers for assessment for inclusion. These papers were allocated to the following topic areas as shown in Table 1.

***Mycoplasma* species/ Research category**	***Mycoplasma mycoides* subsp. *mycoides* (2012-2023)**	***Mycoplasma bovis* (2012-2023)**	**Other mycoplasmas affecting cattle (2015 – 2023)**	**Mycoplasmas affecting swine (2015-2023)**	**Mycoplasmas affecting poultry (2015-2023)**
Biology of the pathogen	10	33	9	34	43
Diagnosis	19	89	18	59	68
Pathogenesis	11	59	11	85	98
Immunology	7	50	1	39	21
Geographic distribution and epidemiology	94	124	48	133	160
Prevention and control	48	95	4	163	140
Total	**189**	**450**	**91**	**513**	**530**

Table 1: Veterinary mycoplasmas peer-reviewed publications, sub-divided by topic area.

These studies formed the main structure of the report and were supplemented by 24 recently published studies retrieved from the PubMed database on 10th May 2023. Additional literature searches were performed during writing to provide appropriate citation for all material and, where needed, useful background. Studies were selected for inclusion based on the authors' impressions of their potential impact within the field and their quality and novelty. More recent studies were given priority within the report. In total, 1,446 studies, reports and resources are referenced herein.

Introduction

The veterinary mycoplasmas comprise a large group of species and subspecies with widely varying clinical significance and host/tissue tropisms. The most pathogenic of these organisms can cause severe diseases, spanning respiratory disease, mastitis, arthritis, conjunctivitis, and reproductive disorders in economically important livestock (primarily chickens, cattle and swine). Mycoplasma-associated diseases of livestock incur substantial losses through reduced productivity (e.g., lower average daily weight gain, reduced milk or egg production, etc.), direct mortality, treatment and control costs, and in some cases, culling of infected animals. These costs are felt in livestock herds and flocks worldwide; while some pathogenic veterinary mycoplasmas are endemic on in limited geographical ranges, many are present across the globe and are therefore very difficult to control, let alone eradicate. Meanwhile, the widespread overuse of antimicrobials to treat mycoplasma-associated livestock diseases continues to promote the development of antimicrobial resistance in mycoplasmas and other bacterial species.

The study of many veterinary mycoplasmas is, in general, a challenging process. Most of these organisms are difficult to culture, making their isolation and *in vitro* characterisation more difficult compared to other, less fastidious bacteria and subsequently reducing disease detection and surveillance (reviewed in (Dudek et al., 2020)). *In vivo*, several clinically important veterinary mycoplasmas also cause chronic infections and can have synergistic effects with co-infecting bacterial or viral pathogens, complicating analyses of pathogenicity, host-pathogen interactions, and epidemiology. Therefore, while the first veterinary mycoplasma was isolated in 1898 (Nocard et al., 1898), the identification and classification of these organisms remains an active field of research. New potentially pathogenic mycoplasmas have been identified over the past decade (Herndon et al., 2021; Stadler et al., 2020), and even the phylogenetic classification of veterinary mycoplasmas has been the subject of continual updates and some disagreement (Balish et al., 2019; Gupta et al., 2019, 2018; Gupta and Oren, 2020; Thiaucourt et al., 2011). Many gene products of pathogenic mycoplasmas also remain uncharacterised, and significant gaps remain in our understanding of host-pathogen interactions in these diseases. Vaccines are available for many of the most economically important veterinary mycoplasmas, but disease control programmes are often stymied by limited protective efficacy and/or vaccine availability in endemic regions.

These limitations call for a combined research and policy framework that prioritises key questions concerning veterinary mycoplasmas, their host species, and their interactions. Coordination of these efforts is critical, as all areas of veterinary mycoplasma research can have important ramifications for

disease surveillance and control. The past decade has seen significant progress in our understanding of the biology and epidemiology of veterinary mycoplasmas, alongside the development of promising new diagnostic techniques, control methods, and vaccine candidates. This report provides updates on key advances in our understanding of and ability to control the most important veterinary mycoplasmas, aiming to establish the avenues of future investigation that will most effectively promote disease management and loss reduction in domestic livestock.

Mycoplasma mycoides subsp. mycoides

Introduction

Contagious bovine pleuropneumonia (CBPP) is a notifiable respiratory disease of cattle and water buffalo that is caused by infection with *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). Thought to have been documented in Europe as early as the mid-1500s, this is not a new disease; however, increased trade also increased the spread of CBPP, leading to reports of infection across the USA, Africa, Asia and Australia in the early-to-mid 19th century (Dupuy et al., 2012). Since then, CBPP has been well-controlled in many regions due to effective monitoring programmes, strict stamping-out policies, movement restrictions, and in some cases, the use of vaccination: the last documented case in Europe was in 1999, and China, the USA, Australia and India are also now officially CBPP-free (for a full list and map of CBPP-free countries, see (WOAH, 2022a)). However, the disease persists in Asia and remains endemic in sub-Saharan Africa (with the exception of South Africa), with an estimated (in 2006) total annual loss of 40 million GBP (47 million USD) across 12 countries (Tambi et al., 2006). This impact is keenly felt at the local level, where a more recent study in Kenya showed that CBPP could cause a family keeping cattle to lose more than twice its annual income, due to vaccination, post-vaccine reactions, treatment costs, morbidity and mortality (Kairu-Wanyoike et al., 2017).

The continuing spread of *Mmm* and its associated economic losses make CBPP the greatest infectious threat to African cattle since the formal elimination of rinderpest in 2011 (USDA, 2017), but the disease surveillance, containment, and mitigation strategies necessary to eventually eliminate CBPP are currently lacking (Jores et al., 2020). Over the past decade, research into the biology of *Mmm* has opened many new avenues, including the development of diagnostics, vaccines, and other control measures, that may substantially boost our ability to control CBPP within endemic regions and prevent its spread to other regions. Effective application of these new data and methods, however, will require greater prioritisation of CBPP by government policymakers. Previous programmes for CBPP control in Africa have mainly relied on international funding due to declining animal health budgets in Africa (reviewed in (Tambi et al., 2006)), and their eventual discontinuation has left CBPP largely uncontrolled within much of its endemic zone (reviewed in (Alhaji et al., 2020)). As a result, the threat CBPP poses to the security and livelihood of millions of cattle owners in Africa must not be underestimated, nor should the benefits (in food security, international trade, etc.) of adequately controlling this disease (Alhaji et al., 2020).

In this section, we will describe some of the most compelling research on *Mmm* and CBPP that has been conducted between 2012 and early 2023. These findings cover the basic biology and molecular mechanisms of *Mmm*, its pathogenesis and immune evasion in host animals, the epidemiology and control of CBPP and other fields crucial for understanding this disease and limiting the damage it causes. We then close by listing the remaining gaps/future research priorities that we believe will be most important for expanding these fields.

Literature review

Biology of the pathogen

Mmm, the causative agent of CBPP, was the first mycoplasma to be isolated in culture, with its identification in 1898 confirming prior knowledge of the contagious nature of CBPP (Nocard et al., 1898; Saraya, 2016). Within genus *Mycoplasma*, *Mmm* is included in the "*Mycoplasma mycoides* cluster" alongside several other pathogenic subspecies responsible for clinically significant infections of livestock (Pettersson et al., 1996). Like all mycoplasmas, *Mmm* is an obligate parasite and is among the smallest known free-living organisms, with a minimum diameter of only 300 nm (Maniloff and Morowitz, 1972) and a 1,211 kb genome (Westberg et al., 2004). Mycoplasmas also lack a cell wall, rendering them pleomorphic and naturally resistant to beta-lactams and other antibiotics that target components of cell wall synthesis (Mitchell et al., 2012).

Molecular typing and genome sequencing

Mmm has low sequence diversity compared to other members of the *Mycoplasma mycoides* cluster, which has been attributed to its relatively recent emergence (Dupuy et al., 2012; Fischer et al., 2012). Notably, however, *Mmm* appears to exhibit higher diversity in Africa relative to historical isolates from pre-eradication Europe, suggesting the presence of understudied evolutionary pathways (Dupuy et al., 2012). Studies of genome sequences from new *Mmm* isolates are important for tracking these pathways and monitoring the geographical spread of the pathogen within its enzootic zone. Prior to 2015, GenBank lacked *Mmm* genome sequences representing virulent circulating strains from Africa (Fischer et al., 2015). Newer studies have begun to address this, and the ongoing development and proliferation of sophisticated next-generation sequencing techniques has facilitated the analysis of numerous *Mmm* genomes over the past decade. These include the Australian strain Gladysdale (Wise

et al., 2012), the Italian strain 57/13 (Orsini et al., 2015) and the sequencing and microscopic analysis of the African challenge strains Afadé and B237 that provided new insights into the bacteria's evolutionary history – these two strains exhibited protrusions similar to the attachment organelle of *Mycoplasma pneumoniae*, but their genomes lacked homologs of the cytoadhesion proteins used by *M. pneumoniae* to create this structure, suggesting the presence of an alternative adhesion pathway (Fischer et al., 2015). Other recently published African *Mmm* genome sequences include the vaccine strain T1/44 (Gourgues et al., 2016) and the Nigerian strains APF9 and AP108 (Di Federico et al., 2019). The sequencing and analysis of *Mmm* genomes will continue to be an important aspect of their characterisation, laying the foundation for in-depth structural and functional studies of the proteins involved in *Mmm* biology and its similarities and differences relative to other mycoplasmas.

Proteomics and gene characterisation

The outer surface of *Mmm*, being the pathogen's point of contact with target cells, is a natural target for such studies of its functionality and fundamental molecular biology. Characterisation of the surface proteome of nine European and African *Mmm* strains identified 44 lipoproteins or cytoplasmic membrane-associated proteins expressed by all the strains, comprising a "core *in vitro* surface membrane-associated proteome" of promising targets for vaccine/diagnostic development and future studies of host-pathogen interactions (Krasteva et al., 2014). The authors of this study note, however, that there may be significant differences between the *in vitro* and *in vivo* surface proteomes of *Mmm*, and they reported relatively low agreement between their 44 identified core surface proteins and previous studies of the *in vivo* serological response to purified *Mmm* antigens (Krasteva et al., 2014). This research group subsequently conducted a more extensive study specifically on the high-pathogenicity African field strain N6 and the low-pathogenicity vaccine strain KH3J, aiming to define new targets in both the surface and intracellular proteomes for targeted studies of individual proteins; they ultimately identified ~31% of the predicted proteome, including 145 previously unreported proteins (Krasteva et al., 2015). Such studies provide valuable roadmaps for further research into *Mmm* proteomic functionality, host-pathogen interactions, and the determinants of pathogenicity.

Alongside large-scale proteomics research, smaller-scale in-depth studies have continued to clarify previously unknown capabilities and functional pathways with important consequences for understanding CBPP and developing effective control strategies. Following the observation that *Mmm* strain Afadé produces two types of colony on solid medium – an "opaque" variant that produces a capsule and a "translucent" capsule-less variant that secretes cell-free exopolysaccharides (Bertin et

al., 2013; Gaurivaud et al., 2004) – these modes were shown to facilitate different bacterial functions *in vitro* and *in vivo*, with the absence of a capsule reducing serum resistance but increasing adherence and antimicrobial resistance (Gaurivaud et al., 2014). The stress-induced switch between these forms was therefore hypothesised to be an adaptive response to different microenvironments, allowing the pathogen to modulate its virulence during the different stages of CBPP (Gaurivaud et al., 2014). These authors later examined the exopolysaccharide secretion by the strain Afadé translucent variant in greater detail, finding that despite their tiny size, the *Mmm* cells secreted extracellular vesicles of highly variable size during culture (Gaurivaud et al., 2018). Analysis of these vesicles' membrane-associated proteins revealed several that had previously been linked to pathogenicity and host-pathogen interaction, suggesting that this secretion pathway may play an active role in *Mmm* infectivity (Gaurivaud et al., 2018).

These studies indicate the broad range of research questions and experimental techniques that have been applied to the study of *Mmm* over the past decade. Even after 125 years of research into the causative agent of CBPP, many aspects of its molecular biology remain only partially understood, and further acquisition of complete genome sequences in conjunction with large- and small-scale proteomics studies will be required to complete the picture of *Mmm*'s core functionality.

Diagnosis

CBPP diagnosis is a crucial part of disease surveillance and control. Pathological findings in suspected cases of disease can provide a presumptive diagnosis (Di Provvido et al., 2018), but laboratory testing is then required to confirm the presence of *Mmm* from nasal swabs or discharges (live animals) or from pleural fluid or tissue samples (necropsy), with polymerase chain reaction (PCR) considered the method of choice for molecular validation of a CBPP diagnosis (reviewed in (Francis et al., 2015a)). World Organisation for Animal Health (WOAH)-recommended serological tests (e.g., competitive ELISA and immunoblotting tests) are also suitable for herd-level diagnosis (reviewed in (Di Teodoro et al., 2020)).

DNA diagnostics

Although highly sensitive, laboratory-based PCR and real-time PCR assays require skilled personnel and laboratory equipment, making them inherently unsuitable for pen-side use. This is a particular

problem in the remote rural areas that comprise much of the CBPP-endemic region of Africa, where access to laboratory services is often severely limited (Vudriko et al., 2021). These restrictions have encouraged the development of isothermal amplification assays that do not require a thermocycler, making them far easier to deploy under field conditions. A new photometric loop-mediated isothermal amplification (LAMP) assay kit was reported to allow detection of *Mmm* DNA directly from crude fluid samples, requiring only a battery-powered mobile device to run the assay (Mair et al., 2013). The sensitivity of LAMP assays for CBPP diagnosis has been compared favourably against serology and even PCR (Enyaru et al., 2012), encouraging further development of isothermal tests for field applications.

Serological diagnostics

Serological assays are generally less sensitive compared to molecular amplification techniques – importantly, no single serological test can reliably detect all CBPP-infected animals, and multiple tests must therefore be applied in parallel to achieve maximum sensitivity (Marobela-Raborokgwe et al., 2003; Sidibé et al., 2012). However, these assays are generally faster and simpler and are thus much more amenable to pen-side diagnostic applications. In Nigeria, a comparison of the dot blot and complement fixation diagnostic tests reported that the dot blot could serve as a reliable, affordable means of presumptively confirming CBPP in slaughtered cattle with lung lesions (Egwu et al., 2012). The latex agglutination test has also been used for on-farm diagnosis in Nigeria (Okaiyeto et al., 2013), though its sensitivity and specificity have been compared unfavourably against the complement fixation test (Odongo et al., 2013). ELISAs have been used for CBPP diagnosis for many years (Le Goff and Thiaucourt, 1998), and Heller *et al.* developed a cocktail ELISA with diagnostic sensitivity and specificity comparable to WOAH-recommended assays – transferring this assay onto a commercially produced lateral flow test platform allowed it to be used for rapid proof-of-concept field diagnosis (Heller et al., 2016).

Sampling strategies and other developments

Training local personnel in the use of diagnostic techniques is necessary for expanding the range of CBPP surveillance and promoting appropriate sample collection and analysis. In Botswana, annual proficiency testing begun in 2010 has dramatically increased CBPP diagnostic skill among participating laboratories (Modise et al., 2018), and a dedicated smartphone app (*VetAfrica-Ethiopia*) was piloted

in Ethiopia to assist student practitioners in diagnosing CBPP and other livestock diseases (Beyene et al., 2017).

Pathogenesis

The respiratory disease caused by *Mmm* infection is characterised by severe bronchopneumonia and pleural effusion in the acute/sub-acute stages, which develops to include the emergence of pulmonary sequestra in chronically infected cattle (reviewed in (Di Teodoro et al., 2020)). Following inhalation of aerosolised pathogen from nearby infected animals, cattle may take anywhere between three weeks and six months to manifest the disease; once they do, CBPP is associated with high morbidity and a variable mortality rate of up to 90% in a naïve and unvaccinated population (reviewed in (Di Teodoro et al., 2020)).

Advances in knowledge of CBPP pathogenesis are hard-won due to the lack of a small animal experimental model and a relative dearth of physiologically relevant *in vitro*/*ex vivo* tissue culture systems. Some progress has been made in understanding the pathogenesis of *Mmm*, but many questions remain. Large animal experiments are costly to conduct and are often carried out in resource-limited settings, restricting the range and type of analyses that can be performed. Collaborations with other laboratories have proven productive, and the emergence of validated *ex vivo*/*in vitro* model systems to investigate the interaction of *Mmm* with host cells represent important steps forward.

In vivo *studies*

CBPP is most commonly induced in cattle by intratracheal instillation of *Mmm.* However, studies have now highlighted the impact of route of experimental challenge, demonstrating that endo-tracheal infection does not fully replicate the features of CBPP seen in animals infected naturally or by contact with experimentally infected cattle (Lutta et al., 2017; Scacchia et al., 2011). These findings are critically important for the planning of experiments seeking to understand host-pathogen interactions, and researchers should aim to avoid the endo-tracheal challenge model wherever possible. Contact challenge is of course more costly and has the major limitation that you cannot be certain of the point of infection; yet, aerosol challenge methods are successfully used to model other bovine diseases, and

there is encouraging evidence that this approach could be similarly standardised and applied to CBPP (Sacchini et al., 2020).

As CBPP becomes established, cattle typically develop pleural effusion from which *Mmm* can also be isolated. The first proteomic characterisation of both the fluid and the mycoplasma within it has yielded interesting insights: firstly, this study detected expression of almost all *Mmm* proteins involved in glycerol import, which culminates in H_2O_2 production, in the pleural effusion, as well as of enzymes involved in synthesis of the potential virulence factor, capsule polysaccharide; from the host side, alongside acute-phase-response- and inflammation-related proteins, the authors detected molecules involved in antigen processing, ubiquitination, and proteasome degradation (Weldearegay et al., 2016). It would be of great interest to compare the *in vivo* proteome generated in this study to the various *in vitro* datasets of *Mmm* protein expression, to assess the extent to which the *in vitro* data are able to inform on the infection of the natural host.

Meanwhile, Li & Wang *et al.* studied the changes associated with host tropism and virulence in *Mmm*, reporting their adaptation of pathogenic strain BEN-1 to rabbits over the course of continuous passaging for 468 generations (during which the bacterium lost immunogenicity in cattle but gained virulence in rabbits). Analysis of strains that emerged during this process revealed a total of 59 specific *Mmm* genes that were involved in host adaptation to rabbits or virulence or immunogenicity in cattle (Li et al., 2016).

In vitro *studies*

Di Teodoro *et al.* reported significant progress in the development of a physiologically relevant tissue culture system in which to study *Mmm* pathogenesis: they showed that bovine respiratory tissue explants can be successfully used to study some aspects of early pathogenesis during CBPP, while noting that further improvements to the model will be required to dissect the local immune response (Di Teodoro et al., 2018a). Their findings led them to suggest that *Mmm* may not be the only relevant source of tissue-damaging H_2O_2 during infection; alongside, activated immune cells were also found to produce H_2O_2 (Di Teodoro et al., 2018a), which indicates the need for further immunological studies of this phenomenon.

Recent work also investigated the use of precision-cut bovine lung slices as an *in vitro* model for understanding the pathogenesis of CBPP. By comparing the *in vitro*-infected lung slices with lung

sections from experimentally infected cattle, the authors concluded that the model was able to recapitulate key features of the disease, including destruction of lung tissue at high doses of *Mmm* (Weldearegay et al., 2019). Comparing these *ex vivo*-infected lung slices with lung slices generated from naturally infected cattle would allow us to more fully understand the applicability of this model.

Additional insights into the early stages of host invasion have come from the application of new tools to meet the challenge. Aye *et al.* utilised flow cytometry to measure the adherence of eight distinct strains of *Mmm* to a range of primary cell types from adult cattle and from foetal lung; the results confirmed the preference of the pathogen for adult lung epithelial cells, consistent with the presence of a specific (yet unidentified) receptor that is lacking or expressed at lower levels on other cell types and in the lungs of foetal cows (Aye et al., 2015). The same group went on to exploit a panel of anti-*Mmm* monoclonal antibodies that inhibited the pathogen's binding to bovine lung epithelial cells, identifying capsular polysaccharide and 2-oxoacid dehydrogenase acyltransferase as important targets used by *Mmm* for cell adhesion (Aye et al., 2018). These studies also provided important insights into the humoral immune response to *Mmm* and are discussed in this context in the Adaptive immunity section.

Despite earlier work showing that some strains of *Mmm* are able to form biofilms on agar which significantly increases their resistance to peroxide, detergent and heat (McAuliffe et al., 2008), and may contribute to pathogenesis, we did not find any studies following up on this phenomenon.

Immunology

CBPP can occur as acute, sub-acute and chronic forms, each with their own distinct pathological features, which are likely mirrored in the host's immune response. Moreover, responses to reinfection are likely to be different: accordingly, unpicking the complexity of the bovine immune response to the various facets of CBPP is an ongoing challenge.

Over the past ten years, progress has been made in understanding the role of T cells in primary infections and reinfections in cattle, and in starting to dissect the interaction of *Mmm* with cells of the innate immune system. Of note, researchers have also uncovered key pieces of information that will support further progress: previously, Scacchia *et al.* showed that significant immunological differences existed between individual animals infected experimentally by either contact exposure or

endotracheal intubation (Scacchia et al., 2011), and some subsequent studies have taken this into account; alongside, the work of Rodrigues *et al.* using whole blood transcriptome analysis showed that local reactions in the lung cannot be inferred from blood samples (Rodrigues et al., 2015), unlike for *Mycobacterium bovis* infection (Rhodes et al., 2000). These studies strongly caution against inference between experimental/natural infection, and extrapolation from findings made in other respiratory pathogens.

Although there remain many hurdles before we have a complete understanding of the immunology of *Mmm* infection, work in this area must be considered a high priority for future research: full understanding of the interaction of the pathogen with the host immune system is a pre-requisite for rational vaccine design and/or the development of novel therapeutics. In turn, gaining this understanding will require comprehensive, accurate and rigorously conducted immunological studies, using the latest tools available and considering the insights into improved experimental design that have been reported in recent years.

Immunogenetics

While it has long been known that CBPP susceptibility varies markedly according to breed (Masiga, 1972), age (Masiga and Windsor, 1978) and immune status of the host, to our knowledge, there have not been any research reports over the past decade concerning the identification of the host factors underpinning resistance or susceptibility to *Mmm* infection.

Innate immunity

Mmm most often enters a host via the airways; therefore, alveolar macrophages are likely to be the first line of defence. While an early study showed that bovine alveolar macrophages incubated *ex vivo* with *Mmm* produced pro-inflammatory TNF-α and nitrogen monoxide (Jungi et al., 1996), later work revealed that monocyte-derived macrophages incubated *in vitro* with the *Mmm* exopolysaccharide galactan produced anti-inflammatory IL-10, and not TNF-α or IL-12p40 (Totté et al., 2015). The authors of the latter study suggested that galactan may thus moderate the inflammation caused by *Mmm*, which could contribute to immune escape; alternatively, this difference could reflect the different cell types and culture conditions used in the two studies. A more recent study, reported in preprint form, showed that bovine monocyte-derived macrophages did secrete TNF-α during incubation with high (500-1000) MOIs of *Mmm in vitro,* but not at a lower (100) MOI: the authors suggested that, as the

higher MOIs were associated with phagocytosis-independent uptake of *Mmm* by macrophages, this could indicate a role for autophagy in TNF-α production by these cells (Totté et al., 2022). The authors also excluded a role for host complement in either blocking *Mmm* adhesion to macrophages or increasing its uptake, while neutralising antibodies increased both uptake and killing of the mycoplasma (Totté et al., 2022). Given the earlier differences between studies using alveolar macrophages and monocyte-derived macrophages, further work is needed to determine the extent to which the *in vitro*-derived cells recapitulate features of primary bovine lung macrophages.

Although the conclusions of the *in vitro* studies are yet to be fully resolved, there is convincing *in vivo* evidence for an important role of TNF-α in CBPP. As discussed in more detail below, Sacchini *et al.* found that high levels of TNF-α in the serum correlated with more severe disease in cattle experimentally infected by intra-tracheal intubation (Sacchini et al., 2012). Furthermore, Sterner-Kock *et al.* experimentally infected a group of *Bos indicus* cattle with *Mmm* and analysed immune cell numbers and the frequency of TNF-α-, IL-1β- and IL-17A-producing cells in their lung tissue at 30 days post-infection (dpi) (Sterner-Kock et al., 2016). They found that CBPP lesions exhibited higher levels of all three tested cytokines compared to lung regions from uninfected controls (Sterner-Kock et al., 2016). Taken together, it seems that TNF-α is consistently induced both locally, at the primary infection site in the lung, and systemically, where higher levels of the cytokine may contribute to, or be a consequence of, pathology.

Neutrophils and lymphocytes are both present within the interstitial space of the CBPP-affected lungs of cattle experimentally infected by intubation at 30 dpi, as are macrophages that contain *Mmm* antigen (Sterner-Kock et al., 2016). Neutrophils may be particularly important in this regard, as a recent study showed that *Mmm* can adhere to, and significantly increase the production of reactive oxygen species (ROS) by bovine polymorphonuclear cells *ex vivo* (Di Teodoro et al., 2018b); this could exacerbate the cell and tissue damage caused by ROS during CBPP (reviewed in (Pilo et al., 2007)). Moreover, bovine neutrophils incubated with *Mmm ex vivo* expressed high levels of IL-1β, IL-8 and COX-2 and 5-LOX, as well as rapidly inducing iNOS, in the first minutes and hours after exposure to the mycoplasma (Di Federico et al., 2020).

Adaptive immunity

Both humoral and T cell-mediated responses are thought to be important in determining protection/recovery from, or susceptibility to, *Mmm* infection, but studies have historically reported

somewhat contradictory results: while early work linked $CD4^+$ T cells and levels of IFN-γ produced by PBMCs *ex vivo* with protection during primary infection (Dedieu et al., 2005a), subsequent studies did not fully support either the correlation between IFN-γ production *ex vivo* and disease severity (Jores et al., 2008) or the idea of a major role for $CD4^+$ T cells in protection from acute disease (Sacchini et al., 2012, 2011). Moreover, a comparison of TNF-α, IFN-γ, IL-4 and IL-10 levels in samples of plasma from experimentally infected cattle that were either depleted of $CD4^+$ T cells from 6 dpi or not, did not reveal any consistent differences in these cytokines between the two groups (Sacchini et al., 2012). The authors did uncover a possible correlation between high levels of TNF-α and more severe disease in the three most-affected animals, and they also noted the general absence of IL-4 in infected animals, a peak in IL-10 at 1-2 weeks post-infection and a peak in IFN-γ at 3 weeks post-infection (which was significantly higher in the absence of $CD4^+$ T cells) (Sacchini et al., 2012). Early T cell depletion did not affect levels of IgG2 or IgM antibodies in plasma, but it did lead to lower levels of IgG1 and IgA later in infection; meanwhile, when animals with severe or mild disease were compared, there was a strong statistical association between high IgG1 in the first two weeks post-infection (and to a lesser extent high IgG2 and IgA) and severe CBPP in the four animals affected (Sacchini et al., 2012). Taken together, these studies show that the absence of $CD4^+$ T cells after the first week of infection has little impact on the levels of the cytokines measured, but the levels of antibodies that may be associated with worse disease are higher in the presence of $CD4^+$ T cells. The validation of these results under more field-like conditions would be a useful goal for future studies, assessing the impact of T cell depletion at the point of infection and measuring the concentrations of a broader range of cytokines/immune mediators/cell populations in the blood and lungs of infected animals.

While the role of $CD4^+$ T cells during the acute phase of primary infection seems to be at best dispensable, and at worst pathogenic, there is some evidence of their importance in establishing the immune memory that is associated with recovery from CBPP and perhaps from reinfection. Early work by Dedieu *et al.* suggested that IFN-γ-secreting memory T cells in the lymph nodes were important for protection of recovered animals (Dedieu et al., 2006); this was supported by Totté *et al.* who examined the cells from lymph nodes draining the lungs of cattle during or after experimental infection with *Mmm* and found evidence of strong recall proliferation of memory $CD4^+$ T cells, accompanied by Th1 cytokine production and T cell-dependent memory B cell proliferation in recovered animals (Totté et al., 2008). These studies highlight the potential differences between the responses detected in blood and those in the draining lymph nodes, urging caution in generalising the conclusions based on one to the situation in the other.

Alongside, several studies have also advanced our knowledge of the role of B cells during CBPP. At 30 dpi, Sterner-Kock *et al.* found that the pulmonary lymph nodes of *Mmm*-infected animals exhibited follicular hyperplasia and had activated germinal centres, while uninfected controls did not (Sterner-Kock et al., 2016). However, an earlier study suggested that high antibody titres during infection might rather more be linked to immunopathology (particularly vasculitis) than to recovery (Schieck et al., 2014), supporting previous work showing the potential negative role of post-vaccination immune complexes in CBPP (Mulongo et al., 2015). Subunit vaccine studies have also generated considerable insights into potentially protective versus pathogenic *Mmm* antigens (see Vaccines section).

Despite the possible immunopathology induced by antibodies during acute infection, understanding the neutralising antibody response is an important step towards the design of effective vaccines against CBPP. Building on their previous work dissecting the specificity of the adherence mechanisms used by *Mmm* to attach to bovine lung epithelial cells (Aye et al., 2015), Aye *et al.* went on to screen a panel of 80 murine monoclonal antibodies specific for *Mmm* and identified 13 clones able to significantly – but not completely – inhibit binding to primary bovine lung epithelial cells, of which one also directly inhibited the growth of the mycoplasma in culture (Aye et al., 2018). This same clone had previously been reported to bind an *Mmm* capsular polysaccharide (Schieck et al., 2016), and Aye *et al.* went on to define the specificity of several others: most notably, they found that three of the clones recognised 2-oxoacid dehydrogenase acyltransferase, which is part of the pyruvate dehydrogenase (PDH) complex that is important for the survival of *Mmm* (Aye et al., 2018). Early studies showed that this protein is immunogenic in experimentally infected cattle (Jores et al., 2009), but more work is needed to understand its functional role *in vivo* and whether targeting this antigen could represent a rational step forwards for vaccine design.

The importance of testing the effect of antibodies specific for *Mmm* antigens is also highly relevant if exacerbation of disease by vaccine-induced immunity is to be avoided. Following earlier work by Hübschle *et al.* that showed the potential of immunising cattle with *Mmm* lipoprotein Q (LppQ) to exacerbate disease (Hübschle et al., 2003), Mulongo *et al.* conducted another study aiming to refine and extend our understanding of this phenomenon (Mulongo et al., 2015). The authors found that immunising cattle three times with LppQ in Freund's adjuvant elicited levels of antibodies that were associated with the development of kidney lesions that appeared to be caused by immune complexes in over 70% of animals challenged with *Mmm* (Mulongo et al., 2015). This is particularly interesting given that anti-LppQ antibodies are present in cattle after natural or experimental infection with *Mmm*

(Abdo et al., 2000), leading to the question of whether antibody responses to this lipoprotein contribute to immunopathology in naturally infected animals.

Lastly, Sterner-Kock *et al.* also reported that IL-17 is present in CBBP lung lesions of experimentally infected cattle at 30 dpi (Sterner-Kock et al., 2016). As studies in mice and humans (reviewed in (Luo et al., 2021)) show that this cytokine can be associated with the pathology of mycoplasma respiratory infections, this finding warrants further investigation.

Despite earlier work showing the potential of the local IgA response to contribute to protection, no work has been done on this.

Maternal immunity

There have not been any notable advances published in this area since 2012.

Immune evasion

Given that animals may recover from, succumb to or become carriers of *Mmm,* it is reasonable to speculate that the pathogen has evolved mechanisms to prevent effective clearance in every case. While these mechanisms are far from completely elucidated, some progress has been made.

Firstly, there is evidence from early *ex vivo/in vitro* studies that *Mmm* can induce apoptosis (Dedieu et al., 2005b) or unresponsiveness (Dedieu and Balcer-Rodrigues, 2006) in bovine leucocytes. In support of this, a more recent study found further evidence of systemic immunosuppression in the blood of contact-infected cattle. Rodrigues *et al.* conducted a comprehensive time-course analysis of global gene expression in whole blood samples from cattle before, during and after acute infection with *Mmm,* finding evidence of widespread downregulation of gene expression, most notably affecting cell-to-cell signalling and cellular functions pathways (Rodrigues et al., 2015). However, the authors noted that this picture does not seem to represent local events in the inflamed lung.

Geographic distribution and epidemiology

Mmm spreads between animals via aerosols, and transmission therefore usually requires close contact between infected and susceptible animals. Lacking a cell wall, *Mmm* is susceptible to many disinfectant methods (e.g., commercial cleaners, heat and high or low pH) – its environmental survival is also short (≤ 2 weeks), restricting its routes of transmission relative to hardier pathogens (WOAH, 2020). Transmission via bodily fluids and indirect transmission (from contaminated surfaces or fomites) appears to be mostly negligible (reviewed in (Di Teodoro et al., 2020)). Long-range transmission is rare but possible under favourable climatic conditions (WOAH, 2023a) and has been reported in past outbreaks (Giovannini et al., 2000), though illegal movement of cattle remains a far more common cause of international transmission events. The significance of transmission from chronically infected animals, which harbour *Mmm* in pulmonary sequestra, is also not completely understood, though it is thought unlikely to play a major role in CBPP spread and maintenance (EFSA Panel on AHAW et al., 2022).

Despite these limitations, *Mmm* is highly contagious via direct transmission within and between cattle herds, facilitating high prevalence and subsequent mortality in endemic areas. CBPP currently remains endemic in sub-Saharan Africa, where it is considered one of the most economically important diseases of livestock in the region, and outbreaks in the 20th century demonstrated the ability of *Mmm* to spread rapidly to other continents if left unchecked (Alhaji et al., 2020; Di Teodoro et al., 2020; WOAH, 2023a).

Global situation

The Americas

There have not been any notable findings published in this area since 2012.

Europe

There have not been any notable findings published in this area since 2012.

Africa

CBPP is widely prevalent across a wide swathe of western, central and eastern Africa. The WOAH Global Framework for the Progressive Control of Transboundary Animal Diseases (GF-TADs) reports that the area of spread and the incidence of outbreaks appear to be increasing, with the latest

reported outbreaks occurring in the Gambia, Niger and Namibia (Mbiri et al., 2020; WOAH, 2023b). Lack of access to vaccines and other veterinary resources allows CBPP to spread rapidly in many regions of Africa, and Zambia and Namibia are currently the only WOAH members with endorsed official CBPP control programmes (WOAH, 2023a). Seroprevalence and abattoir surveillance studies also continue to report high levels of disease burden across this region (Abusara and Abdelgadir, 2014; N'Goran, 2020; Noah et al., 2015; Séry et al., 2015; Yansambou et al., 2018; Zerbo et al., 2021), though methodological challenges in low-income countries (e.g., in sampling design, sample transport and accuracy of diagnostic tests) must be considered when assessing the accuracy of reported data (Lewerin et al., 2018). Endemic CBPP has been described as a "disregarded" disease where insufficient data and lack of surveillance lead to disinterest from donors and researchers, leading in turn to lack of surveillance and reliable data-gathering (Roger et al., 2017). The current status of CBPP control in Africa is further discussed in the Control section below.

In Nigeria, CBPP causes losses of >2 billion USD annually (FAO, 2004) and reported seroprevalence is generally high, with animal-level estimates ranging from approximately 10% to 56% in different states (Ankeli et al., 2017; Anyika et al., 2021; Jamilu et al., 2018; Olorunshola et al., 2020; Suleiman et al., 2015a). Reported CBPP seroprevalence is also highly variable Ethiopia (reviewed in (Abdela and Yune, 2017)), which houses the largest livestock population on the continent (Endalew and Wakene, 2021; Mekuriaw and Harris-Coble, 2021), and the disease appears to be particularly concentrated in the lowland regions where many mobile pastoral cattle herds are kept (Mamo et al., 2018). Numerous analyses have been conducted on the risk factors associated with seroprevalence, but these studies rarely assess the same parameters and often reach different conclusions regarding the significance of factors including cattle age, breed and body condition (Daniel et al., 2016; Kassaye and Molla, 2012; Mamo et al., 2018; Minda et al., 2017; Negash and Dubie, 2021).

Asia, the Middle East and Oceania

CBPP epidemiology in Asia appears to have changed little over the past decade. The outlook from the continent's most populous nations is positive: China remains CBPP-free after eliminating the disease in the 1980s (reviewed in (Jores et al., 2020; Xin et al., 2012)), and India has also maintained its CBPP-free status (EFSA Panel on AHAW et al., 2022). Elsewhere in Asia, the situation is more complicated. Many countries do not report to the WOAH (Xin et al., 2012) and/or lack surveillance programmes against CBPP (EFSA Panel on AHAW et al., 2022; WOAH, 2020). A recent study reported increasing CBPP seroprevalence in northeast Malaysia, but the causative *Mmm* was unable to be isolated (Zarina et al., 2016).

Surveillance is similarly limited in the Middle East, and little research has been conducted there to investigate CBPP epidemiology. Afghanistan is one of the few countries in the region where a large-scale study of CBPP prevalence has been performed, finding no evidence that it is present there (Bahir et al., 2017). In Pakistan, a recent series of clinical and seroprevalence studies reported the presence of CBPP in several districts of Punjab province, near the border with India (Anjum et al., 2020, 2019b, 2019a), but official notifications were not made to the WOAH (EFSA Panel on AHAW et al., 2022).

Surveillance, transmission and risk factors

The predominance of rural, mobile livestock farming systems in many endemic regions of Africa limits the applicability of standard surveillance methods and epidemiological studies, which often rely on regular, standardised data collection from statistically significant population sizes. As with other endemic livestock diseases of Africa, participatory epidemiology has proven a valuable tool for collecting qualitative data on local knowledge, disease prevalence and control measures among smallholders in Africa, who are disproportionately affected by CBPP (Attoh-Kotoku et al., 2018; Baluka et al., 2014; Chenais and Fischer, 2018; Onono et al., 2014). Many such studies have been conducted over the past decade in the endemic countries of Africa including Nigeria (Alhaji and Babalobi, 2016; Geidam et al., 2013; Jamilu et al., 2015), Ethiopia (Bahiru and Assefa, 2020), Kenya (Iles et al., 2019, 2022; Kairu-Wanyoike et al., 2013; Waithanji et al., 2019b), and Uganda (Baluka et al., 2013). These studies have identified numerous sociocultural risk factors influencing *Mmm* transmission, including borrowing/loaning of cattle, sharing of water sources, response to droughts and the extensive management system widely practiced among pastoral cattle herds.

Nigeria is home to a large percentage of Africa's cattle population. Infected animals in that country are regularly sent to abattoirs for slaughter (Nwankwo et al., 2019; Sada et al., 2021), and these infections often go undetected. Investigations of working conditions and management in Nigerian slaughterhouses have found a lack of water and electricity, non-existent hygiene, insufficient inspection of animals and indiscriminate disposal of hazardous animal waste combining to produce a dire outlook for the spread of CBPP within Nigeria's meat production system (Bello et al., 2015; Njoga et al., 2023). Nigeria has continued to conduct mass vaccination campaigns against CBPP using the T1/44 vaccine, but frequent shortages and lack of funding severely limit their efficacy (Majekodunmi et al., 2018).

Vaccination is also often met by strong resistance from pastoral communities. More than 80% of Nigerian cattle are thought to be kept by such communities (Suleiman et al., 2015b), where they play a vital role in food security and income generation. Many pastoralists appreciate the dangers presented by CBPP (Majekodunmi et al., 2018), but this is not universal (Nuvey et al., 2023) and cooperation with vaccination campaigns remains very low overall (Alhaji et al., 2020; Billy et al., 2015; Sada et al., 2015). Importantly, vaccine prices are often unaffordable for pastoral farmers, and veterinary services are inaccessible by many communities; these factors encourage the use of ethnoveterinary practices for CBPP control, ranging from the feeding of medicinal plants to the topical application of kerosene or spent engine oil (Alhaji and Babalobi, 2015; Basheir et al., 2012; Soladoye et al., 2016; Waithanji et al., 2019a).

Wildlife

There have not been any notable findings published in this area since 2012.

Control of the disease

The mainstays of an effective CBPP prevention and control programme are vaccination, treatment, movement restrictions and culling of infected animals (the latter two relying on effective surveillance and diagnosis). However, an opinion article published by Jores *et al.* in 2013 noted that, alongside the shortfalls of the currently available vaccines, the WOAH-recommended diagnostic assays had limited sensitivity in defining the disease status of individual animals, while movement control and destruction of infected animals was impractical in the absence of public support and in the face of mobile production systems, limited veterinary services and little-to-no financial compensation for the loss of the animals (Jores et al., 2013). Perhaps unsurprisingly, data from the WOAH revealed a marked lack of progress in CBPP control in the decade between 2010 and 2020 (Jores et al., 2020).

Given the obstacles to establishing an effective CBPP control programme, standard “field practice” relies more heavily on the somewhat controversial use of antimicrobial drugs such as tetracyclines, macrolides or quinolones. Studies have shown their potentially beneficial effects at the herd (reviewed in (Rweyemamu et al., 2000)), and individual (Muuka et al., 2019; Otina et al., 2022) levels, while in parallel there are concerns that this practice increases antimicrobial resistance risk, sequestra

formation and the number of carriers and may mask clinical signs of disease (FAO, 2004; Niang et al., 2010).

More effective control of CBPP will require action at the international, national, and local levels to overcome these obstacles; the development of improved vaccines remains probably the most important step in increasing the efficacy of control strategies in endemic regions, and some progress towards this aim has been made. Modelling studies have already indicated key parameters for improved vaccines and diagnostic tests (Ssematimba et al., 2015), and may help pave the way towards rational design of next-generation tools in the fight against *Mmm.*

Policy

As noted in the Epidemiology section above, the effective control of CBPP in Africa is hampered by a multitude of inter-related challenges, including under-funded veterinary services leading to poor or no surveillance, low sensitivity diagnostic testing, sub-optimal vaccines and vaccination regimens and a lack of enforcement of control programmes (reviewed in (Alhaji et al., 2020)). Multiple studies have attempted to dissect and propose solutions to these various challenges.

In the many areas lacking centrally organised and funded control programmes, the task of CBPP management most often falls to individual farmers; therefore, understanding the factors motivating or dissuading them from various control measures is an important area of research. Through structured interviews with Nigerian Fulani pastoral herdsmen, Suleiman *et al.* identified the importance of information from trusted sources (the local veterinarian or other herdsmen who had controlled the disease) and good access to affordable veterinary services (Suleiman et al., 2018). In the Narok region of Kenya, education of pastoralist farmers on CBPP control measures was also identified as a priority, as was a strategy to reduce post-vaccine adverse reactions, which were stated as a major factor in pastoralists' decisions not to revaccinate; alongside, farmers expressed a strong preference for vaccinating at specific times of year and together with vaccines against other diseases (Kairu-Wanyoike et al., 2014). Together, these studies highlight the importance of a strong veterinary infrastructure; private sector involvement may help, as shown in parts of Zambia (Muuka et al., 2013), and is recommended by the WOAH as a means of delivering high-quality government-subsidised veterinary services and training for CBPP control (Onono et al., 2017). Alongside, the implementation of an animal identification system would be a relatively low-cost intervention that could markedly assist efforts to control CBPP (Muuka et al., 2012)

Therapeutics

Mmm is susceptible to several types of antimicrobial drug, among which oxytetracycline (Muuka et al., 2019, 2017), gamithromycin and tulathromycin (Muuka et al., 2019) have all been shown to significantly reduce disease severity; in a recent study, tulathromycin treatment also led to *Mmm* clearance in 90% of animals (Muuka et al., 2019). Several studies highlighted the importance of the pharmacodynamic properties of antimicrobials in determining their efficacy against *Mmm*, arguing for the preferential use of drugs with a longer half-life, such as tulathromycin (Mitchell et al., 2013a, 2013b, 2012). These studies evidence a potential role for careful and regulated use of new-generation macrolides – specifically tulathromycin – in the management of CBPP; however, at present the unregulated use of antimicrobial drugs for CBPP risks the emergence of resistant strains and cannot be recommended. Moreover, the suboptimal use of antimicrobials that lessen disease severity but allow residual *Mmm* to survive may also decrease detection of the disease at slaughter, thereby inadvertently contributing to the spread of CBPP.

Alongside the testing of macrolides for CBPP, several studies have reported trials of other synthetic or natural compounds, aiming to assess their use against *Mmm.* Plant-derived traditional remedies are easily available, low-cost, and readily accepted by local communities: proper assessment of their anti-mycoplasma activity is therefore warranted. Following their previous *in vitro* screening study that identified preliminary signs of anti-mycoplasma activity in crude extracts from five traditional medicinal plants used in Kenya (Kama-Kama et al., 2016), Kama-Kama *et al.* went on to test the anti-*Mmm* properties of eight potentially bioactive compounds isolated from *Solanum aculeastrum* and *Piliostigma thonningii* (Kama-Kama et al., 2017). They found that the individual compounds exhibited little or no growth-inhibiting activity against *Mmm in vitro* under the test conditions. This difference with the previous findings could be due to the extraction procedure, or the bioactive compounds present in the crude extract may have worked in concert to induce the previously reported anti-mycoplasma effects. Safety and efficacy studies of these extracts in the field would provide relevant data on their potential as anti-CBPP agents.

Vaccines

Currently available CBPP vaccines are based on the egg passage-attenuated *Mmm* T1/44 strain, either with or without further passage to induce streptomycin resistance (the T1-SR strain) (See also **Error!**

Reference source not found.). These vaccines rapidly induce detectable antibody responses in ~66-75% of cattle after a single immunisation, but levels drop quickly: a large study in the Gambia found that just one third of animals tested positive for anti-*Mmm* antibodies after three months (and 15% has been positive before the immunisation) (Secka et al., 2015). Another study in Kenya showed that 60% of vaccinated cattle that were challenged by intubation developed lesions, compared to 90% of unvaccinated animals, though the lesions were less severe after vaccination (Nkando et al., 2012).

As well as short duration of immunity and incomplete protection from disease, the attenuation of the vaccine strain is not robust; a degree of virulence remains including the retained ability to produce cytotoxic H_2O_2, which is likely to be the cause of the common and relatively severe vaccine site reactions (Mulongo et al., 2013a). These vaccines also require cold-chain storage after reconstitution, which is often problematic. Therefore, an improved vaccine should ideally be safe, extend the duration and extent of protection to around 18 months post-immunisation (Ssematimba et al., 2015), be protective after a single immunisation, be affordable within the means of the current socioeconomic status in CBPP-endemic areas and not require cold-chain storage.

While the field engages with the development of more effective vaccines, it is important to note that, even with the shortcomings of the conventional vaccine, annual herd immunisation was deemed an effective control measure by the WOAH (WOAH, 2018) and was a mainstay of the successful CBPP eradication campaign in Australia in the 1960s (Newton and Norris, 2000), as well as in China with a locally made vaccine in the 1950s (Xin et al., 2012). That said, the situation in Africa is socioeconomically and politically very different, and mathematical modelling somewhat disagrees with the WOAH's conclusion that annual vaccination as it stands will be effective in controlling the disease in sub-Saharan Africa (Ssematimba et al., 2015). Coverage is a key factor in any model vaccine, with a critical minimum of 80% for eradication to be considered possible; yet many African countries are unable to achieve even half this level with the current CBPP vaccines (reviewed in (Dudek et al., 2021)). Therefore, the success of any novel vaccine, or an improved conventional vaccine, will similarly depend on increased vaccine uptake and coverage, as well as complimentary control measures.

Current vaccines

As noted above, studies of the current commercially available vaccines confirm the limited protection, post-vaccinal adverse reactions and short duration of immunity that are elicited (Mwirigi et al., 2016a; Nkando et al., 2012). Attempts to increase the efficacy of the conventional vaccine have been mixed: Nkando *et al.* tested the hypothesis that the immunogenicity of the current vaccine could be improved

by modifying the medium used to grow the vaccine strain, but these authors observed that previous findings of increased viability in the laboratory (March et al., 2002) did not extend to increased protection from challenge in cattle experimentally infected by intubation (Nkando et al., 2012).

Other research has asked whether the current immunogenicity can be retained, but the safety profile improved, by using inactivated *Mmm* in place of the attenuated T1/44 strain. Mwirigi *et al.* compared conventional T1/44 vaccine with heat- or formalin-inactivated *Mmm* strain Afadé, combined with complete or incomplete Freund's adjuvant, respectively, finding that three immunisations with the inactivated vaccines elicited worse or comparable levels of protection compared to a single immunisation with the conventional vaccine (Mwirigi et al., 2016a). Therefore, while the inactivated vaccine route might yet offer important advantages, further study is needed to define the optimal composition of the vaccine, the immunisation regimen, and the duration of protection.

Alternative strategies for vaccine improvement might emerge from a more complete understanding of the immune response to T1/44. Totte *et al.* combined data from groups of cattle at different research sites that had been immunised with the same dose of T1/44 once, T1-SR twice a month apart, or T1/44 three times at annual intervals, and compared their T cell responses to *Mmm* with those of naïve unvaccinated animals (Totte et al., 2013). They found that measurable *Mmm*-specific $CD4^+$ T cell proliferation and IFN-γ production only occurred in the animals that had been vaccinated three times, and also detected $CD4^+$ T cell-dependent proliferation of non-$CD4^+$ T cells (which were not identified in this study) and the presence of both T effector and central memory cells within the proliferating compartment (Totte et al., 2013). These data are important, given the association between the emergence of *Mmm*-specific $CD4^+$ T cells and recovery from CBPP (Dedieu et al., 2006; Totté et al., 2008).

Novel vaccines

Various strategies have been trialled in mice and in cattle to assess potential novel vaccine candidates.

Inactivated vaccines

There have not been any notable advances published in this area since 2012.

Live-attenuated vaccines

There have not been any notable advances published in this area since 2012.

Subunit vaccines

Studies of the adaptive immune response during CBPP (see Adaptive immunity) reveal the presence of protective as well as pathogenic responses to *Mmm*, both of which are likely to be stimulated by immunisation with the live-attenuated organism. Therefore, alongside the general advantages offered by subunit vaccines (safe, inexpensive, and quick to manufacture), here there is the key potential to focus immunity on those antigens most relevant for protection, while avoiding those linked with immunopathology. The challenge remaining is to identify the optimal antigen or combination of antigens, as well as defining the optimal adjuvant to shape the immune response and ensure sufficient immunogenicity.

Trials of subunit vaccines against CBPP have met with mixed success. Mulongo *et al.* hypothesised that antibodies targeting l-α-glycerol-3-phosphate oxidase (GlpO), which mediates the oxidation of glycerol-3-phosphate and so the generation of tissue-damaging H_2O_2 by *Mmm* (Pilo et al., 2005), could both protect cattle from infection and also inhibit the activity of GlpO (Mulongo et al., 2013a). However, they found that while three immunisations of recombinant GlpO in incomplete Freund's adjuvant (IFA) did induce specific antibodies in cattle, these antibodies neither inhibited the enzyme's activity *in vitro* nor protected the animals from experimental challenge (Mulongo et al., 2013a). Notably, monoclonal antibodies raised in mice *did* reduce H_2O_2 production by *Mmm in vitro* while those produced in the immunised cattle appeared to exacerbate the disease (Mulongo et al., 2013a).

Following the identification of GlpO as an immunopathological antigen, Mulongo *et al*. also conducted subunit vaccination with the N-terminus of LppQ from *Mmm* to confirm its role in inducing immunopathology: indeed, they found that immunised animals suffered worse disease, likely due to the formation of immune complexes that caused glomerulonephritis (Mulongo et al., 2015). Shortly after, Mwirigi *et al.* published their findings on the ability of two immunisations with an adjuvanted mixture of *Mmm* capsule polysaccharide conjugated to ovalbumin to reduce the severity of CBPP by almost two thirds, with antibody titres and protection comparable to that elicited by the commercially available vaccine (Mwirigi et al., 2016b).

Further progress towards identifying antigens for inclusion or exclusion in novel CBPP subunit vaccines was made by a pair of studies that screened a larger number of proteins. Perez-Casal *et al.* used reverse vaccinology to define a set of 66 candidate *Mmm* proteins based on their predicted exposure on the surface of the mycoplasma, their likelihood of being involved in adhesion and their recognition by serum from infected cattle; they then tested their immunogenicity *in vivo* (Perez-Casal et al., 2015).

The authors vaccinated groups of cattle with adjuvanted pools of four or five proteins and confirmed the ability of the candidates to elicit antibody responses that were detected by ELISA; however, the proteins failed to induce detectable levels of recall proliferation in peripheral blood mononuclear cells (PBMCs) restimulated *ex vivo* (Perez-Casal et al., 2015). Building on this work, Nkando *et al.* repeated the vaccination experiment with an additional boost after three weeks, and then challenged the groups with *Mmm* administered intra-tracheally two weeks later (Nkando et al., 2016). Here, the use of the complement-fixation test to detect antibody responses indicated that immunisation with the protein pools induced seroconversion at one-to-two weeks only in a minority of the animals and did not induce any specific antibody responses to the pooled proteins that lasted until the point of challenge. Despite this, animals in two of the vaccine groups exhibited significantly less severe lung lesions, and in one of the groups it was not possible to culture *Mmm* from samples of their lungs or pleural fluid at six weeks post-challenge; alongside, several of the vaccinated groups exhibited worsened immunopathology (Nkando et al., 2016). Taken together, these two studies identified several candidate proteins warranting further investigation as components of a subunit vaccine: MSC_0431 (a prolipoprotein), MSC_0136 (a hypothetical lipoprotein), MSC_0816 (a variable lipoprotein) and MSC_0160 (the translation elongation factor Tu). The proteins in groups showing enhanced immunopathology should also be investigated as potential determinants of pathogenicity.

Vectored vaccines

Building on earlier work identifying *Mmm* LppA as a B cell antigen (Cheng et al., 1996), which is also recognised by $CD4^+$ T cells associated with recovery from CBPP (Dedieu et al., 2010), Carozza *et al.* generated a non-replicative human adenovirus 5 (Ad5) vector expressing a truncated form of LppA and used it to immunise mice twice (Carozza et al., 2015). The authors found that the vaccine elicited a strong specific antibody response after a single administration, which was followed by a Th1 type cell-mediated immune response characterised by the production of IFN-γ, TNF-α and IL-10 by splenocytes restimulated *ex vivo* (Carozza et al., 2015). These data are a highly promising basis on which to conduct further studies in cattle, in which the mucosal response and duration of protection could be carefully assessed; further, the incorporation of additional potentially protective antigens identified in subunit vaccine studies could also be trialled to increase efficacy.

Adjuvants

There have not been any notable advances published in this area since 2012.

Conclusions

The studies highlighted here are representative of the current state of research into the biology of *Mmm* and the diagnosis, management and control of CBPP. Over the past decade, substantial strides have been made in these fields, including the sequencing and genomic analysis of new *Mmm* isolates, the identification of potential virulence and immune evasion factors *in vitro* and *in vivo*, an increased focus on epidemiology and surveillance within understudied endemic areas of Africa and the testing of new vaccines to limit the spread of disease in those regions. However, many questions remain unanswered, and much remains to be done to ensure that future efforts to control CBPP find greater success than they have in the past. In particular, the development of control programmes and application of new developments in *Mmm* control and vaccination have been "painfully slow" in Africa, with corresponding consequences for economic and political security (reviewed in (Olorunshola et al., 2017)).

The geographic range of CBPP continues to expand, and the possibility of this pathogen spreading internationally is never negligible. The past decade has seen increased focus on *Mmm* as a pathogen that is capable of threatening cattle populations worldwide if it remains uncontrolled; hopefully, the next decade will continue advancing our understanding of CBPP while applying new disease-monitoring technologies and control measures directly to endemic zones of Africa. Even as more advanced diagnostic and surveillance techniques continue to be developed, international cooperation will be required to ensure that these new resources can be deployed to the remote, resource-limited regions where they are most needed to limit the economic impact of CBPP on vulnerable populations.

Future research priorities

Based on the available literature, expert opinion, and previously published reviews (Jores et al., 2020), we suggest that the following areas of research into *Mmm* should be considered high priority:

Biology of the pathogen

- *Development of a reproducible and robust cattle challenge model, ideally aerosol-based, utilising a low passage field isolate, cattle over one year of age, and with a defined dose of* Mmm*; and applicable to low-resource settings*
- *Continuing collection of complete* Mmm *genome sequences, especially from virulent African strains*

Diagnosis

- *Development and validation of rapid, cost-effective diagnostic assays able to be performed in the field without laboratory equipment*
- *Increased training (on PCR, ELISA, etc.) for veterinary personnel in low- and middle-income countries*

Pathogenesis

- *Verification of surface-localised virulence factors, both in vivo and ex vivo*
- *Generation of* Mmm *mutants based on attenuated M. mycoides subsp. capri model and their testing in vivo as live-attenuated vaccine candidates*
- *Extension of current immunological knowledge, both local and systemic, following natural infection or vaccination against* Mmm*, taking advantage of multi-omics technology to generate valuable insights*
- *Characterisation of the Willem's reaction following vaccination against* Mmm *to support rational design of vaccines without this drawback*
- *Assessment of the potential role of anti-LppQ antibodies in immunopathology*
- *Understanding of responses of respiratory epithelia to* Mmm *infection (e.g., cilia loss, necrosis/apoptosis, metaplasia)*
- *Studies to assess the importance of* Mmm *biofilm formation in the field*

Immunology

- *Standardisation of studies analysing risk factors for CBPP transmission and maintenance, including the incorporation of local sociocultural and/or climatic variables*
- *Characterisation of the local IgA response and its role in protection*
- *Studies to understand the role of maternal immunity in early protection/interference with vaccination of*
- *Immunogenetics of resistance/susceptibility to* Mmm
- *Assessing whether blood sampling is useful in understanding T cell responses to* Mmm *or rather lymph node T cells should be assayed as standard*
- *Identification of pathogenic versus protective antigens/antibodies in cattle*

Epidemiology

- *Increased surveillance for* Mmm *in Africa, the Middle East and Southeast Asia*

- *Communication and outreach methods for promoting vaccine usage among farmers in endemic rural areas*
- *Effective outreach and financial assistance programmes for farmers in low- and middle-income countries*
- *Potential transmission by chronically infected animals under different farm management systems*

Control

- *Design of an improved vaccine with an excellent safety profile: in the absence of/while awaiting significant advances in knowledge on pathogenicity and immunology of* Mmm, *existing lead candidates should be prioritised for further testing, optimisation, and commercialisation*
- *Evidence-based requirements for new vaccines to include a duration of immunity of around 18 months, minimal/no induction of vaccine-site reaction, suitability for needle-free or mucosal delivery, DIVA compatibility, and being cheap, safe and easy to produce*
- *Standardisation of vaccine challenge studies towards a protocol that might include: control unvaccinated group, adjuvant-only group, conventional vaccine group, and two-test vaccine groups (one for challenge and another for duration of immunity studies); the challenge should then be conducted by a standardised aerosol method and disease severity/pathogen titre comprehensively assessed.*
- *Standardisation of immunological readouts for vaccine studies to increase value; assays could include specific immunoglobulin titre, specific T cell proliferation including information on T cell phenotype and cytokine production (ideally by multi-colour flow cytometry), and lung IgA response.*

Mycoplasma bovis

Introduction

Mycoplasma bovis (*M. bovis*) is one of the most pathogenic members of the *Mycoplasma* genus (Calcutt et al., 2018; Francoz et al., 2015) and is predominantly a pathogen of cattle, where it can inflict a wide array of clinical manifestations that significantly impact animal welfare and productivity. One of the most important members of the bovine respiratory disease (BRD) complex, *M. bovis* causes chronic pneumonia and polyarthritis syndrome (CPPS) (Krysak, 2006), and it is a major source of mastitis in dairy cows (reviewed in (Gelgie et al., 2022)). *M. bovis* can also cause other nonspecific manifestations including arthritis, otitis, meningitis, keratoconjunctivitis, endocarditis and abortion, making it very challenging to positively identify the presence of this pathogen without laboratory diagnostics. There are few commercial vaccines available against *M. bovis* in most of the world, and diseases associated with this pathogen are often poorly responsive to drug treatments because of the natural properties of mycoplasmas and a rising trend in antimicrobial resistance (reviewed in (Lysnyansky and Ayling, 2016)). In addition to cattle, *M. bovis* is capable of infecting buffalo and bison, and infections of sheep, goats, swine, deer, chickens and pigs have also been reported ((Spergser et al., 2013); reviewed in (Calcutt et al., 2018)).

Altogether, these properties make *M. bovis* an extremely difficult pathogen to control. Originally identified in the United States in 1962, *M. bovis* spread quickly via international animal movements that facilitate direct contact transmission among cattle, and it has now been detected in every major cattle-rearing country (reviewed in (Dudek et al., 2020)). As it is already pervasive across the globe, *M. bovis* is not subject to WOAH regulations (Dudek et al., 2020), but the consequences of infection are substantial. Because many *M. bovis* infections are chronic and/or subclinical, treatment and productivity costs tend to be higher compared to other pathogens in the BRD complex (reviewed in (Maunsell et al., 2011)). Comprehensive estimates of the total economic burden of *M. bovis* are rare, but older estimates indicate the scale of the problem (Nicholas and Ayling, 2003): total losses due to *M. bovis* were estimated to be at least 144 million euros (~237 million euros adjusted for inflation in 2023) in Europe, while *M. bovis*-associated mastitis alone cost an estimated 108 million USD (~195 million USD adjusted for inflation in 2023). The lack of options for controlling this pathogen make it one of the most important mycoplasmas of livestock today, and substantial economic benefits could be reaped by expanding our ability to detect, contain and treat it.

This section will cover a representative subset of the research conducted on *M. bovis* and its clinical manifestations from 2012-2023, including the molecular biology and epidemiology of this pathogen, its typing and diagnosis, current strategies for control and ongoing research into vaccines and effective antimicrobial treatments.

Literature review

Biology of the pathogen

Like other mycoplasmas, *M. bovis* is a tiny obligate parasite with low genomic G+C content and lacks a cell wall, rendering it inherently resistant to beta-lactams and other cell wall-targeting antibiotics (reviewed in (Ammar et al., 2021)). It is also naturally resistant to several other classes of antimicrobials including polymyxins and sulfonamides, adding to the difficulty of controlling *M. bovis*-associated disease in lieu of a vaccine (reviewed in (Lysnyansky and Ayling, 2016)).

Considering these disadvantages, monitoring and surveillance of *M. bovis* are two of our most powerful tools for limiting the spread of infection. Genomic analyses are an important component of these approaches, allowing researchers to track the spread and evolution of specific *M. bovis* lineages within a region or country. Over the past decade, research into the molecular biology of *M. bovis* has focused primarily on two fields: the typing and/or genome sequencing of new *M. bovis* isolates and the classification of gene functions, many of which remain unknown.

Molecular typing and genome sequencing

Molecular typing is essential for understanding the evolution and population structure of *M. bovis* and the genomic factors involved in its transmissibility, but typing techniques have historically been complicated by subjective interpretation and a lack of standardisation among analysis methods (Pinho et al., 2012; Register et al., 2015). Multilocus variable number tandem repeat (VNTR) analysis (MLVA), which allows a higher degree of comparability by relying on standardised primer sequences and DNA sequencing workflows, has been used to circumvent these issues (Pinho et al., 2012; Spergser et al., 2013). MLVA of *M. bovis* isolates from Israeli cattle identified 35 different VNTR types, some associated with strains originating in Israel and other from strains in cattle imported from Europe and Australia (Amram et al., 2013). A similar study of Hungarian isolates used MLVA to map within-herd

divergence of *M. bovis* strains, where the fine-scale typing of MLVA was ideal for discriminating between closely related strains isolated from single farms (Sulyok et al., 2014b).

At the intermediate scale (i.e., comparing less closely related isolates), multilocus sequence typing (MLST), a similar technique that classifies isolates based on polymorphisms in housekeeping genes rather than on number of tandem repeats (Jenke et al., 2011), is generally considered more suitable for phylogenetic comparisons (Sulyok et al., 2014b). Two MLST methods for *M. bovis* were reported independently in 2015 (Register et al., 2015; Rosales et al., 2015). Rosales *et al.* used their method to identify 35 distinct sequence types (STs) in two main clonal complexes which differentiated most British and German *M. bovis* strains from a more heterogeneous set including European, Asian and Australian isolates (Rosales et al., 2015). The initial report from Register *et al.* was published alongside a corresponding public database for *M. bovis* MLST data (PubMLST) and used a different set of seven loci to distinguish 32 STs from cattle and North American bison (*Bison bison*) (Register et al., 2015). While this study identified four bison-specific and 28 cattle-specific STs, indicating that genetically distinct *M. bovis* populations are responsible for infections in these respective species, the authors later found four STs that were shared between cattle and bison isolates and one shared between cattle and North American deer (*Odocoileus virginianus* and *hemionus*) isolates (Register et al., 2019). This study also confirmed an earlier report identifying *M. bovis* isolates lacking the *adh-1* typing locus used in their original MLST method (Josi et al., 2018), necessitating a revised version of the PubMLST reference method that incorporated the previously validated *dnaA* locus (Rosales et al., 2015) in place of *adh-1* (Register et al., 2020b). MLST can also be combined with MLVA when both intermediate- and fine-scale typing are required (L. Manso-Silván et al., 2013). A comparison of *M. bovis* isolates from Canadian cattle and North American bison employed this combination alongside whole-genome sequencing to identify 10 STs, 20 VNTR types and 40 putative virulence genes among these isolates (Menghwar et al., 2021a; Menghwar and Perez-Casal, 2022).

As sequencing and genomics techniques have continued to improve in precision and efficiency, the molecular typing repertoire has diversified as well. Newer developments include core genome MLST (cgMLST), which analyses alleles from hundreds of genes as opposed to the typical seven housekeeping genes used in standard MLST (Menghwar et al., 2022), and core- and whole-genome variations of single-nucleotide variant (SNV) comparison. Kinnear *et al.* recently compared these phylogenetic methods using 129 *M. bovis* isolates from North American cattle, reporting pros and cons to each approach (Kinnear et al., 2021). cgMLST provided a higher genotypic resolution than standard MLST but lacked ease-of-use; whole-genome SNV demonstrated the highest resolution among the

four methods and was able to type all 129 isolates (compared to 101 for cgMLST and cgSNV), but further development will be needed to improve the intuitiveness of this workflow (Kinnear et al., 2021).

These molecular typing techniques can generate phylogenetic data from only partial *M. bovis* genomic sequences, but whole genome sequencing is also necessary to provide the up-to-date reference sequences required for tracking the evolution and transmission of bacterial lineages (Ambroset et al., 2022; Wise et al., 2011). The genome of *M. bovis* is unusual, with numerous highly repetitive regions and low G+C content even in comparison to other mycoplasmas (Bürki et al., 2015; Razin et al., 1998; Zhou et al., 2014). However, the proliferation and continuing simplification of standardised next-generation sequencing techniques has facilitated the collection of many complete *M. bovis* sequences over the past decade, including isolates from Canada (Menghwar et al., 2021b; Register et al., 2020a), France (Ambroset et al., 2022), Belgium (Bokma et al., 2020d), China (Chen et al., 2017; Shen et al., 2020; Sun et al., 2018), Japan (Morimoto et al., 2019) and Australia (Parker et al., 2016b). In addition to supporting disease surveillance and phylogenetics, complete genome sequences also provide crucial information for subsequent studies of strain-specific pathogenesis and evolution – analysis of the genome sequence of the Chinese Ningxia-1 strain, for instance, identified several suggestive differences from the reference *M. bovis* genome, including variable surface lipoproteins and pathogenicity islands (Sun et al., 2018).

Proteomics and gene characterisation

Genotyping and whole-genome comparison of new *M. bovis* isolates against reference strains provide valuable insights, allowing researchers to associate particular adaptations with specific sites in the genome. However, despite the small size of the *M. bovis* genome, many of its gene products are uncharacterised (Kumar et al., 2020). Genome dynamics and evolutionary adaptations are difficult to map to precise functions. A comparison of the Chinese HB0801 isolate with the established standard PG45 reference strain, for instance, revealed substantial genomic plasticity, including a large chromosomal inversion, a significant reduction in variable surface lipoprotein genes, and numerous smaller insertions and losses within the genome – notably, however, HB0801 and PG45 both exhibited similar pathology in cattle, indicating that these genomic differences had not impacted their virulence (Qi et al., 2012). Prediction of the phenotype of an isolate based on its genotype is an attractive target, but the genetic complexity of *M. bovis* and the myriad interactions between pathogen, host and environmental variables during infection render this a daunting task. In their comparison of four MLST-

and SNV-based genotyping methods, for instance, Kinnear *et al.* reported that none could associate genotypes with phenotypes (Kinnear et al., 2021).

Where such large gaps exist in our understanding of a pathogen's functional components, wide-scale high-throughput methods are important for narrowing the field and identifying genes of particular interest. Sharma *et al.* generated a library of transposon mutants of the PG45 strain, then directly sequenced mutants with insertions in putative membrane nuclease genes (Sharma et al., 2015). Inactivation of the *mnuA* gene specifically abrogated cellular exo- and endonuclease activity, indicating that MnuA is an important bacterial nuclease involved in the acquisition of nucleotide precursors (Sharma et al., 2015). This enzyme was also later shown to aid in immune escape by degrading DNA-containing neutrophil extracellular traps (Mitiku et al., 2018) (see also Immunology).

In a similar high-throughput study, Josi *et al.* generated a library of *M. bovis* strain JF4278 mutants, then applied high-throughput screening and bioinformatics to classify genes as essential vs. non-essential (Josi et al., 2019). Among other findings, this workflow identified a set of non-essential virulence-associated genes, including three factors (the adhesin TrmFO and two previously unknown proteins) associated with a reduced-adhesion phenotype (Josi et al., 2019).

Metabolomics provide complementary data to large-scale genome screening, focusing on the downstream products of functional pathways to identify their unidentified components. Masukagami *et al.* used ^{13}C isotope labelling to track *M. bovis* metabolism in culture; these authors uncovered new functional metabolic pathways and assigned likely functions to previously uncharacterised *M. bovis* genes, including a putative *sn*-glycerol-3-phosphate ABC transporter operon, a phosphotransferase system transporter and a multifunctional phospohoribosyltransferase (Masukagami et al., 2019). These findings are also discussed in the Pathogenesis section below.

Finally, the CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease) genome-editing system has recently been applied to *M. bovis* (alongside *Mmm* and *M. gallisepticum*), with Ipoutcha *et al.* demonstrating proof-of-concept by successfully knocking out the *mnuA* gene in the PG45 strain (Ipoutcha et al., 2022).

Diagnosis

Diagnosis of *M. bovis* is complicated by its various clinical signs (which can easily be confused with those of other bovine pathogens), the presence of subclinical infections, intermittent pathogen shedding and varying test responses observed in animals of different age groups (Penterman et al., 2022; Petersen et al., 2020). Recommendations for high-sensitivity *M. bovis* diagnosis often involve bacteriological culture (accurate, but difficult and time-intensive) and/or the combination of multiple diagnostic tests (which may increase false positive rates) (Manning, 2020). The efficacy of any given test depends on farm-, herd-, and animal-level factors (reviewed in (Parker et al., 2018)), making field validation a vital component of diagnostic development in order to define the conditions under which each test is most effective.

Numerous *M. bovis* diagnostic tests had been published in the research, and some commercialised by 2012, but advancements in sequencing, nucleic acid amplification techniques and serology have been applied over the past decade to produce new or improved tests with higher sensitivity and positive detection rates. Many of these tests have been designed with ease-of-use in mind to facilitate their deployment in remote locations that may lack access to standard veterinary laboratory services. This section will discuss some of these advancements, bearing in mind that combined use of diagnostic tests will continue to be the most effective means of obtaining a definitive *M. bovis* diagnosis.

DNA diagnostics

Isolation and culturing of *M. bovis* is the gold standard for diagnosis, but the slow-growing and fastidious nature of mycoplasmas necessitates the use of highly specialised and time-consuming culture methods (reviewed in (Dudek et al., 2020)), and growth-inhibited or dead bacteria due to antibiotic treatment cannot be detected (Bell et al., 2014). For these reasons, many laboratories use PCR as a fast and accurate alternative for routine diagnosis of *M. bovis* (reviewed in (Parker et al., 2018)), and a recent interlaboratory comparison of different PCR techniques found a high level of reproducibility among methods and labs, supporting a high degree of confidence in PCR data generated under different circumstances (Wisselink et al., 2019). The *uvrC* gene, which encodes a DNA repair protein in *M. bovis*, has long been used as a target for PCR diagnostics because of its conservation among field isolates (Naikare et al., 2015; Rossetti et al., 2010; Subramaniam et al., 1998; Thomas et al., 2004). Notably, however, Register *et al.* reported several *M. bovis* isolates with mutations that caused failure of *uvrC*-targeted PCR, including a single nucleotide polymorphism in a

primer-binding region and a transposase gene insertion within the amplicon (Register et al., 2018a). The *oppD* gene has also been used as a target for molecular diagnosis of *M. bovis*, with associated real-time PCR (qPCR) assays providing high sensitivity and specificity (Loy et al., 2018; Sachse et al., 2010).

As *M. bovis* infections can cause varied and nonspecific phenotypes, multiplex PCR (incorporating multiple primer pairs that amplify distinct target sequences) can be a useful tool for identifying and/or discriminating *M. bovis* against other potential causes of observed clinical signs (Gioia et al., 2016). In 2018, two independent groups reported the development of multiplex qPCR tests for discriminating *M. bovis* from its fellow BRD complex members *Mannheimia haemolytica*, *Histophilus somni* and *Pasteurella multocida* (Loy et al., 2018; Thanthrige-Don et al., 2018). Zheng *et al.* developed a multiplex qPCR assay for differentiating *M. bovis* from four other pathogens (three bacterial and one viral) capable of causing bovine pinkeye (Zheng et al., 2019), and a multiplex qPCR targeting the *rpoB* gene of *M. bovis* was shown to distinguish the bacterium from other mycoplasmas and *Acholeplasma laidlawii* in milk samples with 100% and 93% diagnostic specificity and sensitivity, respectively (Chauhan et al., 2021). DNA Diagnostic A/S developed two commercial multiplex qPCR assays – Pneumo4B and 4V – for the detection and quantification of bacterial (including *M. bovis*) and viral members of the BRD complex, reporting high diagnostic performance by both kits (Pansri et al., 2020). The BioMark HD high-throughput qPCR system has also been used as a lower-cost alternative to multiplex PCR, differentiating *M. bovis* from other respiratory and enteric pathogens in pooled faecal and nasal swab samples (Goecke et al., 2021).

Finally, post-PCR high resolution melting curve analysis has been employed in several studies to effectively discriminate *M. bovis*, and even individual *M. bovis* isolates, based on the 16S gene or 16S-23S intergenic spacer region (Ahani Azari et al., 2020; Ajitkumar et al., 2012; Al-Farha et al., 2018b; McDaniel and Derscheid, 2021).

While highly accurate and reproducible, PCR assays typically require laboratory equipment, trained personnel and cold storage, meaning that they often are impossible to conduct in remote, resource-limited regions (reviewed in (Calcutt et al., 2018)). One common solution is isothermal nucleic acid amplification, which is relatively simple and does not require a thermocycler (reviewed in (Koczula and Gallotta, 2016)), making it ideal for use under field conditions. Conrad *et al.* developed a set of isothermal recombinase polymerase amplification (RPA) assays for multiplex identification of BRD-associated pathogens and detection of antimicrobial resistance genes (Conrad et al., 2020), while two

groups reported their development of *uvrC*-targeting isothermal RPA assays combined with lateral flow strips for greater ease-of-use (R. Li et al., 2021; Zhao et al., 2018).

LAMP is another commonly employed technique for field-capable rapid diagnostic tests, offering performance that is generally equivalent to or slightly greater than RPA, depending on the assay (Howson et al., 2017; Zou et al., 2020). Bai *et al.* developed a *uvrC*-targeting LAMP assay (Bai et al., 2011), and its diagnostic performance was later improved by targeting the *oppD/F* locus and incorporating a loop primer to increase amplification speed (Higa et al., 2016). Meanwhile, a comparison of three LAMP assays targeting the *uvrC*, *gyrB* or 16S rRNA genes of *M. bovis* in milk from mastitic cattle reported the highest sensitivity and specificity from the *uvrC*- and *gyrB*-targeting assays, respectively (Ashraf et al., 2018), and a similar approach combining LAMP with the commercial Loopamp PURE DNA extraction kit achieved comparable performance versus traditional PCR (Itoh et al., 2020). This requirement of a DNA extraction step is an important hurdle for developing pen-side tests aimed at minimising the use of laboratory equipment (Appelt et al., 2019), and continued development of field-useable nucleic acid extraction techniques (and/or amplification diagnostics useable on crude samples) will likely be an important component of future studies.

Serological diagnostics

Serodiagnostics fill a need for rapid, sensitive assays that detect the presence of anti-*M. bovis* antibodies to demonstrate whether a specific cohort or herd has been exposed to this pathogen. For instance, herd-level serodiagnostics can also be particularly effective at identifying herds from which purchasing animals may pose a higher risk of introducing *M. bovis* (Parker et al., 2017a). ELISAs are the most used *M. bovis* serodiagnostic, but their performance is affected by several test- and target-specific factors. Establishing a reliable cut-off value is critical for obtaining optimal sensitivity from diagnostic ELISAs, and this value will vary depending on sample type (Al-Farha et al., 2020). The sample type itself and the stage of infection in tested animals are also important to consider. A recent study of Danish dairy herds reported that milk antibody levels were increased only in mastitic animals, indicating that milk samples can only be used to diagnose animals with *M. bovis* infection presenting with mastitis (Petersen et al., 2018a).

The choice of capture antigen is particularly important for pathogen-specific diagnosis, as many antigens expressed by *M. bovis* are also expressed by other mycoplasmas (reviewed in (Parker et al., 2018)). The variable surface proteins (Vsps) were among the first immunogenic proteins of *M. bovis* to be identified, but their propensity for genetic rearrangements makes them poor targets for

serodiagnostics (Rosengarten and Yogev, 1996). More recent studies have identified immunogenic proteins that are highly conserved and less prone to variation. The *M. bovis* PDHB and P48 proteins have been targeted by indirect and direct ELISAs, respectively, to produce assays with higher positive detection rates relative to comparable commercial kits (Fu et al., 2014b; Sun et al., 2014), and the lipoprotein MbovP579 was later identified as an immunogenic target for highly *M. bovis*-specific serodiagnosis (Khan et al., 2016). Wawegama *et al.* developed an ELISA based on a recombinant fragment of the *M. bovis* membrane lipase MilA, reporting ~94% diagnostic sensitivity and specificity in feedlot cattle under field conditions (Wawegama et al., 2016, 2014). This group observed similar results in later studies that compared the MilA ELISA favourably against commercially available ELISAs (Petersen et al., 2018b; Salgadu et al., 2022a) and in a diagnostic evaluation on bulk tank milk (BTM) samples from Australian dairy herds (Salgadu et al., 2022b).

Several commercial ELISAs for *M. bovis* serodiagnosis are available on the market. Bio-X Diagnostics, for example, produces two commercial ELISAs – the BIO K302 and K260 kits – for detection of antibodies in serum, plasma, and milk samples. Nielsen *et al.* compared the BIO K302 test and PCR in diagnosis from milk samples, reporting that raising the ELISA cut-off value above the manufacturer's recommendation provided higher sensitivity than PCR (~44% versus 37%) while maintaining near-100% specificity (Nielsen et al., 2015). A later comparison of both Bio-X kits against western blotting in the serodiagnosis of experimentally infected cattle was more negative, reporting high specificity but lower sensitivity for the BIO K302 and K260 kits (47% and 28%, respectively) versus western blotting (74%) (Schibrowski et al., 2018a). More recently, IDVet released their ID Screen indirect ELISA for *M. bovis*, and a field comparison of this test against the BIO K302 kit reported substantially higher sensitivity (though several false positives were also observed) (Petersen et al., 2020). Alongside the above-mentioned interlaboratory trial of PCR reproducibility, a corresponding comparison of serological methods reported high consistency between the ID Screen ELISA and western blotting, which offered higher diagnostic precision and accuracy compared to the BIO K302 (Andersson et al., 2019).

While ELISAs comprise the bulk of *M. bovis* serodiagnostics, other technologies have been also been tested for their performance or ease-of-use compared to this standard technique. Fu *et al.* developed an indirect competitive enzyme-linked aptamer assay (using single-stranded oligonucleotides rather than antibodies as the molecular recognition agent) against the *M. bovis* P48 protein, reporting higher positive detection rates from bovine serum compared to commercial ELISAs (Fu et al., 2014a). The simplicity and cost-effectiveness of lateral flow assays (LFAs) has made them an attractive target for

M. bovis serodiagnostics as well: Shi *et al.* developed an LFA using colloidal carbon test strips to detect *M. bovis* antibodies in serum and reported 100% specificity and >97% diagnostic sensitivity under laboratory conditions (Shi et al., 2020), and pen-side validation could confirm the utility of this simple visual assay under field conditions.

Finally, the detection of *M. bovis* outbreaks in American bison around the turn of the millennium (discussed in more detail in Epidemiology) required that serodiagnostic tests be validated in this species as well. Unfortunately, ELISAs developed for cattle exhibited low sensitivity in bison, particularly in animals with low-to-moderate infection levels, but replacing the capture antigen with one derived from bison isolates improved assay performance (Register et al., 2013a).

Sampling strategies and other developments

Efforts have been made to improve the throughput of traditional culture methods for *M. bovis* diagnosis. A recent approach predicted the likelihood of *M. bovis* isolation via C_t values obtained from high-throughput qPCR on direct cultures of deep nasopharyngeal swabs, which ruled out 97.3% of *M. bovis*-negative samples without further processing (Andrés-Lasheras et al., 2020).

The development of third-generation sequencing has also opened new avenues for pathogen diagnostics, facilitating the incorporation of whole-genome sequencing into cost-effective and field-deployable tests. A recent study applied taxon-specific basecalling for the Oxford MinION sequencer and single-use long-read nanopore sequencing to the detection of *M. bovis* field strains, reporting results comparable to those obtained from hybrid (short- and long-read sequencing) approaches (Vereecke et al., 2020). This group later developed a workflow for rapid identification of *M. bovis* in bovine bronchoalveolar lavage fluid using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and compared this assay against nanopore sequencing, reporting higher diagnostic sensitivity but lower specificity versus sequencing (Bokma et al., 2020c, 2021b).

Finally, other studies have aimed to identify biomarkers that could indicate the presence of clinical or subclinical *M. bovis* infections in individual animals. The levels of several inflammation-related microRNAs (miRNAs) were reported to increase significantly in the milk of infected Holstein-Friesian and Foğu Anadolu Kırmızısı cows (Özdemir, 2020); however, a later analysis of the miRNA profiles in tissues isolated from *M. bovis*-infected calves indicated that circulating miRNA levels were unsuitable for assessing disease condition and did not correlate with tissue expression levels (Casas et al., 2022).

A recent study using 16S rRNA sequencing and metabolomics reported significant differences in the nasal microbiota of healthy versus diseased cattle, indicating that the prevalence of various bacterial species and serum metabolites may be useful as biomarkers for different infections and co-infections responsible for BRD (Y. Zhang et al., 2022).

Pathogenesis

Typical infections of cattle with *M. bovis* are associated with caseonecrotic pneumonia, mastitis and arthritis; yet, there are reports of many other tissues being affected including the eye, the ear, the brain meninges, the skin, the heart and the reproductive system (reviewed in (Dudek et al., 2020)). Recently identified less-typical presentations in the field include necrotic pharyngitis (Dyer et al., 2013), placentitis associated with abortion (Register et al., 2013b), the formation of caseonecrotic granulomas in cattle (Suwanruengsri et al., 2022) and the detection of transplacentally transmitted *M. bovis* in aborted bovine foetuses (de Oliveira et al., 2022; Kathrin Hermeyer et al., 2012). *M. bovis* has also been isolated from post-surgical seromas arising after caesarean section (Gille et al., 2016) and from calves with toxic epidermal necrolysis (Senturk et al., 2012).

These broad and varied observations from the field raise many important questions around the pathogenesis of *M. bovis* infections. In the past ten years, researchers have attempted to provide answers by examining animals infected in the field or under experimental conditions, conducting studies in experimental models, and generating data from *in vitro* analysis of infected cells.

In vivo *and* ex vivo *studies*

M. bovis has become increasingly recognised as a key factor in the BRD complex in both adult cattle (T. E. S. Oliveira et al., 2020) and in calves (Antonis et al., 2022; Pardon et al., 2020), and has been detected in cases of acute (Centeno-Martinez et al., 2022; Oliveira et al., 2021; Timsit et al., 2018; Valeris-Chacin et al., 2022) as well as chronic BRD, where is accepted as a causative agent (Becker et al., 2020; Booker et al., 2008; Mehinagic et al., 2019). However, these findings are complicated by the long-known fact that the upper respiratory tract of young calves is often colonised by *M. bovis* in the absence of clinical disease, which may later develop as the pathogen migrates to the lower respiratory tract or middle ear (reviewed in (Perez-Casal, 2020)). More recent analysis of the broncheoalveolar lavage fluid of healthy calves also detected *M. bovis* in almost two thirds of samples – roughly the

same frequency as in calves with BRD – indicating that migration of the pathogen alone does not always lead to disease (Kudirkiene et al., 2021). A preceding study also detected *M. bovis* in lung tissue of diseased and healthy calves, but in this case, 24 out of the 26 *M. bovis*-positive animals exhibited clear signs of disease (Giovannini et al., 2013). These data raise many questions about how *M. bovis* asymptomatic colonisation progresses to frank disease, and whether the factors driving this change are host-derived, pathogen-derived, or perhaps relate to coinfecting microorganisms or local environmental influences. *M. bovis* isolates from diseased versus healthy calves do not appear to significantly differ genetically (Castillo-Alcala et al., 2012), nor do they produce differing amounts of H_2O_2 when cultured *ex vivo* (Schott et al., 2014). Current experimental *M. bovis* exposure models have yet, to our knowledge, been used to study field-like asymptomatic colonisation, which significantly limits our ability to answer key questions about the progression or otherwise to overt clinical disease.

Several studies have shed light on the colonisation of different host organs by *M. bovis*. Meningitis is a rare but serious complication of infection, but it has long been unclear whether this was most likely to result from spread of *M. bovis* through the blood, lymph, directly through a nearby lesion, or via ascending infection along a peripheral nerve. A recent study revealed that direct spread was most likely, showing that in cattle with meningitis, *M. bovis* had likely first infected the palatine tonsil, next moving into the nearby eustachian tube, from where the pathogen could directly penetrate into the brain (Suwanruengsri et al., 2021). Complementing these findings, an earlier study showed that the most likely route of initial colonisation of the tonsil in calves was the consumption of *M. bovis*-containing milk, and that from the infected tonsil, the mycoplasma was able to colonise both the lower respiratory tract and the middle ear (Maunsell et al., 2012).

Infection of the joints by *M. bovis* can occur as a sequelae lung infection (Gagea et al., 2006), and causes a severe inflammatory response that leads to pain, swelling, synovial hyperplasia and osteolysis (Desrochers and Francoz, 2014). Recent studies have shed light on the molecular processes involved. Devi *et al.* uncovered signs of nitritative injury in the joints of calves that had been experimentally intraarticularly inoculated with *M. bovis*, with evidence of production of iNOS and nitrotyrosine by macrophages that were associated with the necrotic tissue lesions (Devi et al., 2014). More recently, Nishi *et al.* found that *M. bovis* arthritis was associated with a massive increase in the proportion of neutrophils within the synovial fluid, which was accompanied by significantly increased expression of cytokines such as IL-1β, IL-6, IL-8, IL-17 and IL-22 at the gene (Nishi et al., 2019) and protein (Nishi et al., 2021b) levels; the authors speculated that IL-1β could promote the bone resorption seen in *M. bovis* arthritis, while IL-6 might drive synovial hyperplasia, and IL-8/IL-17 were likely to attract

neutrophils and leucocytes, respectively. The same group went on to further dissect the interaction with bovine synovial cells by exposing them to *M. bovis ex vivo*, finding that the mycoplasma invaded the cells via clathrin-dependent, caveolin-independent, endocytosis; once inside the synovial cells, *M. bovis* was able to evade killing by antibiotics added to the culture medium (Nishi et al., 2021a).

Increasing our understanding of early events during *in vivo* infections would be greatly enhanced by the development and validation of a cattle infection protocol that reliably induced field-like disease via a natural (or natural-like) inoculation route. When aiming to study *M. bovis* pneumonia, intratracheal (K. Hermeyer et al., 2012; Prysliak et al., 2011) or intranasal (White et al., 2012; Zhang et al., 2014) inoculation has been favoured, but these require large numbers of bacteria to induce clinical disease and the administration route is likely to prove stressful to animals, which could have relevant effects on their susceptibility/disease process. Moreover, earlier studies showed how cattle age/pathogen co-infection status can markedly affect the outcome of these commonly used routes of challenge (Prysliak et al., 2011). Kanci *et al.* reported promising results from a novel aerosol infection method that exposed small groups of calves twice to *M. bovis* in a controlled air-flow chamber and required minimal handling; this method induced disease in all exposed calves with comparable features to *M. bovis* pneumonia reported in the field (Kanci et al., 2017). It would be of interest to establish whether this approach could also replicate asymptomatic colonisation of young animals, which could lead to invaluable insights into the processes determining pathologic progression of *M. bovis* infection.

Cattle are not the only hosts of *M. bovis,* and studies are now showing that bison are highly susceptible to pneumonia and arthritis (Dyer et al., 2008) and to necrotic pharyngitis (Dyer et al., 2013) caused by this pathogen. Register *et al.* showed that isolates of *M. bovis* from cattle and bison are phylogenetically distinct (Register et al., 2015), and also that bison are uniquely vulnerable to *M. bovis* isolates that circulate within this species, while cattle are not (Register et al., 2018b). Earlier work revealed distinct *in vitro* properties of *M. bovis* from bison and cattle, including differences in their abilities to invade lung epithelial or tracheal cell lines, and their interactions with PBMC and alveolar macrophages (Suleman et al., 2016a). Together, these findings point towards important species-specific features of *M. bovis* isolates from bison and cattle, but a fuller picture of the distinct processes or determinants of pathogenicity in each species has yet to emerge.

Finally, it is widely acknowledged that the development of an improved small animal model of *M. bovis* infection would facilitate studies of its pathogenesis and immunology. Towards this aim,

Schneider *et al.* injected the mammary glands of specific-pathogen-free C3H/HeN mice with an *M. bovis* field strain and conducted histopathology and gene expression analysis 24-48 hours later; they found that *M. bovis* injection caused massive neutrophil recruitment and inflammation, accompanied by elevated expression of genes encoding TNF-α, iNOS and IκBα (Schneider et al., 2022). Alongside, the authors exposed murine mammary epithelial cells to live or UV-inactivated *M. bovis*, showing that NF-κB activation was stimulated by both: this differs from both (also contradictory) previous studies of NF-κB activation in bovine mammary epithelial cells (Gondaira et al., 2018; Zbinden et al., 2015), and should therefore be interpreted with caution. Bovine lung organoids may be a better option for understanding the pathogenesis of lung disease caused by *M. bovis*: Archer *et al.* reported preliminary data showing the feasibility of generating such organoids from embryonic lungs (Archer et al., 2021), yet to our knowledge they have yet to be exploited for research into mycoplasma-associated diseases. Similarly, bovine "lung-on-a-chip" technology is beginning to emerge (Lee et al., 2022).

In vitro *studies*

Embryonic bovine lung (EBL) cells are a commonly used tool for the study of *M. bovis* infections. Recent research described their use to identify changes in gene expression elicited by the pathogen: infected cells expressed significantly lower levels of genes associated with metabolic and immune processes, most notably those in the PI3K-Akt-mTOR signalling pathway, which is involved in nutrient metabolism, compared to control cells (X. Wu et al., 2022). The same group previously showed that application of exogenous recombinant *M. bovis* membrane lipoprotein P48, which may participate in the pathogen's adhesion to host cells, was sufficient to induce ER-stress-mediated apoptosis, leading them to speculate on a similar role for this protein *in vivo* (Wu et al., 2021).

EBL cells have also been used in *in vitro* studies aiming to identify individual *M. bovis* virulence/adhesion factors. Zhu *et al.* showed that a novel *M. bovis* cytoadhesin, encoded by Mbov_503, was required for efficient cell adhesion and translocation of the mycoplasma across epithelial cell monolayers (X. Zhu et al., 2020). Alongside, Gao *et al.* showed that fructose-1,6-bisphosphate aldolase is also involved in the adhesion of *M. bovis* to EBL cells (Gao et al., 2018), as are methylenetetrahydrofolate-tRNA-(uracil-5-)-methyltransferase (YongPeng et al., 2017) and P27 (X. Chen et al., 2018). On the host side, Zhang *et al.* recently identified annexin A2 expressed on EBL cells as an important partner for *M. bovis* adhesion and invasion in this system (H. Zhang et al., 2022).

Aiming to shed light on the processes underpinning mammary tissue damage in *M. bovis* mastitis, Liu *et al.* exposed bovine mammary epithelial cells (MEC) to large (MOI of 1000) numbers of *M. bovis* from two different field strains, finding that the mycoplasma induced ROS production *in vitro* leading to mitochondria-mediated apoptosis of MEC (Y. Liu et al., 2020). The extent of ROS production and MEC death varied between strains (Y. Liu et al., 2020), but it was not established whether this related to differences in virulence *in vivo*, which is difficult to assess given the influence of multiple other host factors. Also in MEC, Liu *et al.* provided convincing evidence that after invading the cells, *M. bovis* was able to block the autophagic pathway, preventing acidification of the autophagosomes, and allowing its own survival in cells; if the pathway was reactivated by drug treatment, *M. bovis* was killed within MEC (Y. Liu et al., 2021). A follow-up study led the group to conclude that *M. bovis* elicited knock-down of autophagy by promoting phosphorylation of PI3K-Akt-mTOR (Xu et al., 2022). The lower MOI of 30 used in these studies increases confidence in the conclusions, warranting their follow-up *in vivo*.

Efforts to identify novel determinants of *M. bovis* virulence using genomics and targeted mutation have generated interesting data. Rasheed *et al.* conducted a comparative genomics study between a virulent *M. bovis* field strain and several passage-attenuated variants. This approach revealed changes to 11 genes that were deemed likely to relate to virulence: ascorbate-specific phosphotransferase system enzyme IIB and IIA components, enolase, L-lactate dehydrogenase, pyruvate kinase, glycerol, and multiple sugar ATP-binding cassette transporters, ATP binding proteins, NADH dehydrogenase, phosphate acetyltransferase, transketolase, and a variable surface protein (Rasheed et al., 2017). Other researchers have generated modified *M. bovis* strains with mutations affecting specific genes thought to be involved in pathogenesis. For example, Zhao *et al.* generated *M. bovis* lacking full-length NADH oxidase, which allowed the identification of this protein as being involved in the process of attachment to EBL cells, as well as being required for production of H_2O_2 (G. Zhao et al., 2017). Masukagami *et al.* also used this approach to selectively ablate the functions of predicted nutrient transport genes of *M. bovis*, then compared their metabolic profiles; they found evidence of a surprising level of redundancy within the glycerol transport system, among others, and identified different roles for some genes than predicted *in silico* (Masukagami et al., 2019).

In vitro gene mutation and protein expression studies have also yielded interesting insights into the likely functions of *M. bovis* molecules. Adamu *et al.* showed that *M. bovis* P226 (encoded by the *milA* gene) was surface-exposed and had a range of lipid- and glycosaminoglycan-binding properties, including a carboxyl-terminal that bound and hydrolysed ATP (Adamu et al., 2020).

Immunology

The immune response to *M. bovis* is a primary determinant of the onset, severity, duration, and outcome of disease, and it is particularly challenging to study given the multiple manifestations of the infection and the different tissues that can be affected. The presence of asymptomatic infection in animals has yet, to our knowledge, to be purposefully replicated experimentally and is therefore relatively unstudied (though experimental infection studies do occasionally unintentionally generate an asymptomatic state in one or two animals). Individual animals also vary in susceptibility, and so studies seeking the knowledge required for molecular breeding programmes focusing on *M. bovis*/BRD resistance traits should be encouraged. Earlier work suggested several innate immune loci that could be involved (Neibergs et al., 2014; Tizioto et al., 2015), yet the follow-up outcomes of these findings have yet to be reported.

Most immunological studies since 2012 have focused on immune evasion strategies of the pathogen, which broaden and deepen our understanding, even though many have been conducted *in vitro* or *ex vivo.* However, there are also important advances to report in our knowledge of innate and adaptive immune pathways that could feasibly be used to improve the prospect of designing effective treatments and/or better vaccines. Notably, the discovery of bovine lung-specific NK-lysins, which have bactericidal effects that would be difficult for *M. bovis* to escape (Junfeng Chen et al., 2015; Chen et al., 2016; Dassanayake et al., 2018); and the insights gained from considering the results of several studies on the polarisation of the immune response during infection/vaccination and how this might be linked to pathogen clearance, persistence or exacerbation of disease (Behura et al., 2017; Chao et al., 2019; Ohtsuka et al., 2020; Prysliak et al., 2018).The majority of immunological studies since 2012 have focused on immune evasion strategies of the pathogen, which broaden and deepen our understanding, even though many have been conducted *in vitro* or *ex vivo.* However, there are also important advances to report in our knowledge of innate and adaptive immune pathways that could feasibly be used to improve the prospect of designing effective treatments and/or better vaccines. Notably, the discovery of bovine lung-specific NK-lysins, which have bactericidal effects that would be difficult for *M. bovis* to escape (Junfeng Chen et al., 2015; Chen et al., 2016; Dassanayake et al., 2018); and the insights gained from considering the results of several studies on the polarisation of the immune response during infection/vaccination and how this might be linked to pathogen clearance, persistence or exacerbation of disease (Behura et al., 2017; Chao et al., 2019; Ohtsuka et al., 2020; Prysliak et al., 2018).

Immunogenetics

There have not been any published studies in this area since 2012.

Innate immunity

Innate immunity in the respiratory mucosa is likely to be the first line of defence against *M. bovis*. Among the key cell types involved are alveolar macrophages, which are known to be present in, and around, *M. bovis* pneumonia lesions (K. Hermeyer et al., 2012). Monocyte-derived lung macrophages are also likely to form an important part of the immunological network during *M. bovis* infection. A recent study compared the responses of alveolar macrophages and monocyte-derived-macrophages to *M. bovis in vitro*, and found that while the monocyte-derived cells secreted matrix metalloproteinase-12 and secreted phospholipase A2 (which is involved in cell death), the lung alveolar macrophages did not (Baquero et al., 2021). Whether this represents a difference that can be recapitulated *in vivo* and its relevance for the disease pathway are topics for future investigation.

Alongside macrophage functions, the role of Natural Killer (NK) cells in *M. bovis* is starting to be explored. An important antimicrobial mechanism used by NK cells is the production of NK-lysins; peptides that are electrostatically attracted to the membranes of pathogenic microorganisms but not host cells, and can either directly kill some pathogens, or synergise with other immune mechanisms to render them more effective. The genome of cattle contains four NK-lysin-encoding genes, whereas most mammals possess just one; interestingly, *NK2C* is predominantly expressed in the lung (Junfeng Chen et al., 2015). Chen *et al.* also observed marked upregulation of the *NK2C* gene in the lungs of animals challenged with *M. bovis* compared to controls (Chen et al., 2016), while Dassanayake *et al.* documented significant damage to the plasma membrane of *M. bovis* following incubation with recombinant bovine NK2A or NK2C *in vitro* (Dassanayake et al., 2018). Such antimicrobial peptides are an attractive potential therapeutic option, given their innate specificity for microorganisms and the lack of receptor-mediated-binding, making their action hard or impossible to develop resistance to; it is also possible that selective breeding for increased NK-lysin activity/expression could represent a promising strategy for increased antimicrobial resistance, as shown in genetically modified fish (Huang et al., 2018) and beginning to be explored in pigs (Tong et al., 2022).

As well as studies focusing on the lung, there are several reports of insights into the response of mammary cells exposed to *M. bovis.* In one study, *ex vivo* incubation of primary bovine MEC to high concentrations of *M. bovis* induced expression of IL-6, IL-8 and TNF-α at the mRNA level, as well as Toll-like receptors (TLRs) 1, 2, 3, 6 and 9 at the mRNA and protein levels (J. Yang et al., 2022). This confirmed a previous report of the induction of transcription of TNF-α, IL-1β, IL-6, IL-8, lactoferrin, TLR2, RANTES and serum amyloid A mRNA in MEC exposed to live but not inactivated *M. bovis* (Zbinden et al., 2015). A different study using a bovine MEC cell line and a range of infection levels also found significantly increased expression of IL-1β, IL-6, TNF-α, TLR2 and TLR4, but in this case, only after exposure to killed and not live *M. bovis* (Rodríguez et al., 2015). The same group also exposed PBMC to *M. bovis ex vivo*, detecting significantly increased expression of the genes encoding IL-12p40 and IFN-γ at an MOI of 100, and conversely, proliferation of PBMC only at an MOI of 100 or less (Gondaira et al., 2015). Taken together, these studies show that bovine MEC, at least *ex vivo/in vitro*, have the capacity to initiate programmes of immune gene expression in response to *M. bovis;* whether these pathways are initiated in primary cells under physiological/near-physiological levels of exposure to the pathogen will require additional study to resolve.

Adaptive immunity

Studies in this area have predominantly aimed to characterise the adaptive cytokine and cellular responses of immune cells exposed *ex vivo* to *M. bovis*, or within samples from infected animals, and in some cases to correlate their findings with disease severity. A clear picture of protective immunity has yet to emerge, but advances continue to be made.

Concerning the systemic immune response, Chao *et al.* compared the transcriptomes of PBMC from calves nasally inoculated with either virulent or attenuated *M. bovis,* or medium broth alone; this allowed them to identify potentially relevant gene sets that were associated with disease severity (Chao et al., 2019). Calves exhibiting worse gross and lung scores during infection with virulent *M. bovis* expressed higher levels of *ATP5B*, *JAK1*, *RORB*, *SYK*, *IL-17D*, *IL-23R*, *STAT3*, *GSK3B*, *IL-1RL1*, *ACTG1*, *TP53*, *IL-6*, *IL-21R* and *GATA3*; while those with less severe disease expressed higher levels of *INS, INSR, UBC, TNF, NFKBIA, FOXP3, NOS3, HSPA8, TLR4, BIRC3* and *IFNG* (Chao et al., 2019). Lastly, given the prevalence of IL-17-related genes in the former list, the researchers measured the levels of these transcripts in lungs of infected animals, finding that diseased animals expressed significantly higher levels of *IL-17A, IL-6, IL-21R* and *IL-23R* (Chao et al., 2019). It remains unclear whether these immune signatures are a result of severe disease that drives persistent inflammation with a Th17

component, or whether the immune response of these calves is more permissive to *M. bovis* and results in worse outcomes for the animals.

Further support for the importance of Th-17/IL-17 in the response to *M. bovis* came from Ohtsuka *et al.* who collected blood samples from *M. bovis*-infected calves at the onset of symptoms and either at the end of treatment or at the point of euthanasia, and compared levels of cytokine transcripts between those calves that resolved their symptoms and those that did not (Ohtsuka et al., 2020). They found that lack of resolution of the infection was associated with significantly higher IL-17 levels that were maintained through treatment or until the animal was killed (Ohtsuka et al., 2020). PBMC exposed to *M. bovis ex vivo* similarly increased their expression of IL-17 mRNA, as well as of IL-36A, IL-27, and IFN-γ (Gondaira et al., 2021b). Lastly, an earlier vaccine study aiming to elicit Th-17 responses in fact achieved exacerbation of disease upon challenge, in agreement with the above findings (Prysliak et al., 2018). Taken together, these data provide strong support for further investigation of the role of IL-17 in *M. bovis* infection.

By contrast to Th-17 responses, there is some evidence that Th1 responses are elicited following vaccination with reportedly protective attenuated *M. bovis* strains (Chao et al., 2019) (discussed below, see Live-attenuated vaccines). Enriching this picture further, a detailed transcriptional study that compared lung and lymph node transcriptomes following challenge with different pathogens involved in BRD uncovered evidence of a primarily Th2 response in *M. bovis*-challenged animals as well as identifying common innate pathways activated during infection with respiratory pathogens (Behura et al., 2017). In summary, the above studies paint a picture of Th17/Th2 immunity being a hallmark of more severe and/or persistent infection with *M. bovis*, while features of Th1 immunity appear more likely to be related to protection (at least following vaccination).

Few studies have reported advances in our understanding of the antibody response to *M. bovis*, though the work of two groups has offered insight into the potential role of complement-fixing antibodies in protection from disease. Zhang *et al.* initially reported that rabbit antibodies recognising *M. bovis* membrane proteins were able to initiate complement-mediated killing of the pathogen *in vitro* (Zhang et al., 2019); and, while some bovine antibodies were able to replicate these *in vitro* results, vaccination against the proteins recognised by complement-fixing antibodies did not enhance protection of cattle from *M. bovis in vivo* (Prysliak et al., 2023).

Maternal immunity

There have not been any notable advances published in this area since 2012.

Immune evasion

M. bovis exhibits multiple means of immune evasion, which are strongly implicated in its ability to persist in the host. Most of the proposed pathways have been primarily evidenced *in vitro;* some of these findings are contradictory while others appear to reflect the *in vivo* situation more clearly. The pathways and processes identified warrant further investigation to enhance our understanding of the pathogenesis of *M. bovis* infection and to inform rational vaccine strategies.

It is clear that during disease caused by *M. bovis,* immunosuppression – or at least immunomodulation – occurs *in vivo* (for example (Dudek et al., 2013)). Various immune-suppressive processes have been evidenced *in vitro*: *M. bovis* has both induced (Bürgi et al., 2018; G. Zhao et al., 2021) and inhibited (Maina et al., 2019; Mulongo et al., 2014) apoptosis of bovine monocytes and macrophages, with the reasons for the discrepancy in findings yet unclear. Alongside, other studies showed that *M. bovis* infection of monocytes reduces their ability to produce IFN-γ and TNF-α but increases production of the immunosuppressive cytokine IL-10 (Mulongo et al., 2014); while earlier work identified the ability of *M. bovis* to infect multiple cell types within bovine blood, reducing PBMC proliferation (van der Merwe et al., 2010). The mechanism of this reduced proliferation might also have been identified: Suleman *et al.* uncovered a potentially important role of the immune-inhibitory programmed death-1 (PD-1)/PD ligand-1 (PD-L1) pathway in this process, showing elevated expression of PD-L1 on macrophages from the lungs of infected animals, coincident with increased expression of PD-1 on both $CD4^+$ and $CD8^+$ T cells in the blood, which together correlated with low proliferation of PBMC (Suleman et al., 2018). A more recent study has contributed a further strand of knowledge, showing that *M. bovis* shapes the systemic immune environment to favour the emergence of regulatory T cells and the production of TGFβ, which inhibited the production of the Th1 cytokines IFN-γ and TNF-α by bovine PBMC *in vitro* (Sajiki et al., 2020).

Recent work has also uncovered a potentially significant role for prostaglandin E2 (PGE2)-driven upregulation of the PD-1/PD-L1 pathway. Goto *et al.* initially correlated increased expression of PD-1 on T cells from *M. bovis*-infected cattle with reduced IFN-γ production by their PBMC *ex vivo*, then showed that blocking PD-1/PD-L1 reinstated IFN-γ production by *M. bovis*-stimulated PBMC (Goto et

al., 2017). The same group went on to show that PGE2 was also induced by incubation of PBMC with *M. bovis ex vivo*, as well as finding elevated levels of PGE2 in the blood of infected cattle, both of which correlated with elevated expression of PD-L1 in each setting (Goto et al., 2020). The authors suggested that *M. bovis*-induced PGE2 production by monocytes could increase expression of PD-L1 on bovine T cells, limiting their ability to produce IFN-γ and promoting mycoplasma survival in the host. Further support for the significance of this pathway came from a study by Gondiera *et al.*, who found elevated expression of PD-1, PD-L1 and other immune-suppression/exhaustion genes in mononuclear cells from the milk of experimentally infected mammary glands (Gondaira et al., 2020).

PGE2 is also known to affect innate immune cells, including neutrophils, in other species; and although it is not yet known whether this pathway is relevant in *M. bovis* infection, it is clear that neutrophils are a main target of immune suppression. Jimbo *et al.* incubated *M. bovis* with bovine neutrophils from healthy cattle *ex vivo* and found that the mycoplasma inhibited the production of nitric oxide, increased production of IL-12 and TNF-α, and promoted neutrophil apoptosis (Jimbo et al., 2017). *M. bovis* also appears able to disarm neutrophil extracellular traps (NETs), which are DNA-based structures that represent potent antimicrobial tools in other settings. Bovine neutrophils did not form detectable NETs after incubation with *M. bovis ex vivo*, and the addition of *M. bovis* led to destruction of existing nets in stimulated neutrophil cultures, most likely due to the action of the *M. bovis* nuclease and/or its inhibition of ROS production (Gondaira et al., 2017). These findings were supported by more recent work showing that live *M. bovis* could escape/prevent the formation of NETs, while killed *M. bovis* could not (Gondaira et al., 2021a), and the study by Mitiku *et al.* that revealed the NET-degrading ability of *M. bovis* major membrane nuclease MnuA (Mitiku et al., 2018).

Lastly, a study by Zhao *et al.* found that *M. bovis*, like other mycoplasmas, encodes at least two surface proteins that are able to bind *M. bovis*-specific IgG *in vitro*: they showed that a recombinant version of MBOVPG45_0375 prevented effective binding of the antibody to the mycoplasmal surface, while in concert, recombinant MBOVPG45_0376 could proteolytically cleave IgG (H. Zhao et al., 2021). The extent to which these proteins are involved in *in vivo* immune evasion requires further investigation.

Geographic distribution and epidemiology

Mycoplasma bovis began spreading across the globe in the mid-20th century, with introductions of the pathogen facilitated by burgeoning international trade networks and a lack of understanding of the

opportunistic nature of *M. bovis* infections (reviewed in (Dudek et al., 2020)). Today, anthropogenic factors (e.g., cattle trading, farm management factors and insufficient biosecurity) continue to play a major role in the long-distance spread of *M. bovis* (Aebi et al., 2015; Yair et al., 2020). Even subclinical *M. bovis* infections have been linked with significant decreases in milk yield and quality (Timonen et al., 2017), and the occurrence of these infections facilitates undetected transmission, with subsequent cases of pneumonia, arthritis, clinical mastitis and other morbidities causing economically significant infections in affected cattle populations (reviewed in (Gelgie et al., 2022)). At present, most of the world's major economies have been exposed to *M. bovis* infections for decades, causing widespread productivity losses within the beef and dairy cattle industries (reviewed in (Nicholas, 2011)).

The past decade saw the introduction of *M. bovis* to two countries that had previously been holdouts. Finland reported its first identification of *M. bovis*-infected cattle at the end of 2012, in the wake of increasing outbreaks in Denmark and introduction to Sweden in 2011 (Arede et al., 2016; Hurri et al., 2022), and the pathogen has subsequently spread among the country's dairy herds (Haapala et al., 2018; Tardy et al., 2020). This made Norway the only remaining Scandinavian country that was considered *M. bovis*-free, though a lack of recent publicly available data has been reported in that country (Tardy et al., 2020). In 2017, an *M. bovis* outbreak was reported in New Zealand, though the pathogen had likely already been present there for years (Jordan et al., 2021). New Zealand implemented an *M. bovis* eradication programme the following year, with outbreak delimitation via network and background surveillance still ongoing (Cowled et al., 2022; Ministry for Primary Industries, 2023).

Global situation

The Americas

M. bovis was first identified in the USA, after its isolation from dairy cattle with mastitis in 1962 (Hale et al., 1962). Today, it remains the most prevalent mycoplasma among American cattle herds, causing substantial production losses alone or in co-infection with other bacterial or viral pathogens (Gioia et al., 2021; Lubbers et al., 2017; Soehnlen et al., 2012). *M. bovis* was also commonly identified in a recent study of Canadian beef feedlot cattle with bronchopneumonia (with or without interstitial pneumonia) or acute interstitial pneumonia (Haydock et al., 2023).

M. bovis is also widespread in South America. In Brazil, a recent analysis of bronchoalveolar lavage fluid samples from dairy calves found *M. bovis* to be the second most common BRD-associated

pathogen complex (after *Pasteurella multocida*) in these animals (V. H. S. Oliveira et al., 2020). Interestingly, an earlier study on nasopharyngeal swab samples from steers on beef cattle feedlots did not detect any *M. bovis* (or indeed *P. multocida*) (Headley et al., 2018). Whether this indicates differences in BRD aetiology between dairy calves and beef steers, or is simply attributable to different sample types or diagnostic techniques, is not clear. Meanwhile, molecular diagnostic surveys of milk samples have indicated that *M. bovis* is also a major cause of bovine clinical mastitis in Brazil (Adorno et al., 2021; Junqueira et al., 2020; Salina et al., 2020), and a survey of Brazilian beef cattle reported that *M. bovis* infections may create a favourable environment in bulls for co-infection with the infertility-associated mollicute *Ureaplasma diversum* (Carli et al., 2022).

Similar findings have been reported in Argentina, where Margineda *et al.* published the first report of *M. bovis*-associated bovine pneumonia and polyarthritis in feedlot calves in 2017 (Margineda et al., 2017). Molecular analysis of samples from Argentinian dairy cattle indicated that *M. bovis* is likely secondary to other mycoplasmas (namely *M. leachii* and *californicum*) as a cause of arthritis and mastitis in these animals (Neder et al., 2022). Argentina currently lacks commercially available vaccines against *M. bovis*, making proactive surveillance and outbreak control critical for preventing unnecessary disease-related production losses (Cantón et al., 2022).

M. bovis has also been detected in milk samples from dairy cattle in Chile (Ulloa et al., 2021), though it was less prevalent than *M. bovigenitalium* (discussed in more detail in Other mycoplasmas affecting cattle).

Europe

The abolishment of the EU's milk production quotas in 2015 led to a dramatic expansion in dairy farming, which unfortunately also facilitated increased inter-herd transmission of *M. bovis* and other livestock pathogens (McCarthy et al., 2021). The highest-profile development in *M. bovis* epidemiology in Europe over the past decade was its emergence in Finland, and the majority of published studies from the continent report seroprevalence levels in different countries or states. Below is a discussion of studies from representative countries that illustrate general trends in *M. bovis* transmission and occurrence in Europe over the past decade.

The reported seroprevalence of *M. bovis* is particularly high in Spain (Calderón Bernal et al., 2023) and in Poland (Bednarek et al., 2012; Dudek and Bednarek, 2012; Szacawa et al., 2015), where *M. bovis* infections are common on dairy farms and statistically significant correlations have been observed

between the occurrence of *M. bovis* and of its fellow BRD-associated pathogens *Mannheimia haemolytica* and *Histophilus somni* (Lachowicz-Wolak et al., 2022). Clinical infections have also been reported in European bison (*Bison bonasus*) in Poland (Dudek et al., 2015; Krzysiak et al., 2014).

Meanwhile, *M. bovis* infections in Switzerland and Austria primarily caused pneumonia and subclinical mastitis prior to 2007, when severe clinical mastitis cases suddenly increased in both countries (Bürki et al., 2016). An analysis of *M. bovis* strains circulating within Swiss cattle herds found that they were mostly herd-specific, indicating that this spate of outbreaks had not been due to the sudden introduction of any one specific clone of *M. bovis* (Aebi et al., 2012). A subsequent molecular typing study in Switzerland and Austria classified pre- and post-2007 *M. bovis* isolates into separate lineages, with the newer lineage hypothesised to display higher virulence toward mammary gland cells (Bürki et al., 2016).

M. bovis is also highly prevalent among dairy cattle in the Netherlands, with an estimated true prevalence of ~75% (Veldhuis et al., 2022). A recent study of disease dynamics on 20 Dutch dairy farms during early-stage acute clinical outbreaks found that subclinical infections were common and that *M. bovis* transmitted to cattle of all age groups despite separate housing (Penterman et al., 2022).

M. bovis remains widespread in the United Kingdom, where it was the most frequently identified mollicute in an analysis of diagnostic samples received from ruminant farms between 2005-2019 (Deeney et al., 2021), though it is not currently a notifiable pathogen in the country (Ridley and Hateley, 2018). In neighbouring Ireland, a recent seroprevalence study among dairy herds also confirmed continuing endemicity, with herd size and the number of neighbouring farms found to be significant risk factors for herd-level prevalence (McAloon et al., 2022). Herd size was also identified as a particularly important risk factor in Sweden, where *M. bovis* seroprevalence remains generally low but is concentrated in the south of the country (Hurri et al., 2022).

The epidemiology of *M. bovis* is strongly dependent on local and regional variables (e.g., different farming systems, different trade patterns, etc.) that can be very difficult to predict or control. In Belgium, for example, a recent phylogenomic analysis classified circulating *M. bovis* strains into five separate major clusters, with genetic similarities to Israeli, European and American isolates indicating high levels of transmission across international trade networks and among veal, dairy and beef herds (Bokma et al., 2020d). Conversely, an earlier study reported much lower genetic diversity in the Austrian Alps, which saw successive waves of *M. bovis* emergence and re-emergence between 2005-

2009 before a sudden and dramatic increase in outbreaks in 2010-2011 (Spergser et al., 2013). Here, molecular typing indicated that a single *M. bovis* strain had re-emerged in 2009 and subsequently spread throughout the Austrian Alps region; the authors hypothesised that the unique livestock farming practices of the Alps (involving regular transhumance movements and shared mountain pastures) may have supported this epidemiological pattern (Spergser et al., 2013). Similar results emerged from France, where Becker *et al.* analysed the genetic diversity of collected *M. bovis* isolates using MLST and MLVA (see Biology of the pathogen) supplemented with MALDI-TOF MS to compare their protein expression patterns (Becker et al., 2015). Despite the propensity of *M. bovis* for generating heterogeneous strains, these authors reported a loss in genetic diversity since the 1990s, suggesting the spread of a single clone within France and implicating antimicrobial use in the selection of drug-resistant strains (Becker et al., 2015).

Finally, Fanelli *et al*. conducted a retrospective analysis of the role of *M. bovis* in a string of deadly calf pneumonia outbreaks on Italian dairy farms between 2009-2019, reporting high animal- and herd-level prevalence (~16% and 27%, respectively) and common co-infections with other cattle respiratory pathogens (Fanelli et al., 2021). These authors also observed seasonal morbidity peaks in April and September, positing unknown stress conditions during these periods as a possible cause (Fanelli et al., 2021).

Africa

Few recent studies have addressed the epidemiology of *M. bovis* in Africa beyond small-scale, local-/regional-level analyses, despite the economic impact of BRD across the continent. BRD places a severe burden on cattle farming in Egypt, for instance, and *M. bovis* appears to be particularly widespread, though its exact prevalence in this country has proven difficult to confirm due to inter-study variation and the application of vaccination programmes (Hashem et al., 2022b). The identification of *M. bovis* DIVA (differentiating infected from vaccinated animals) markers and the development of corresponding vaccines would benefit the determination of true prevalence levels (and, thereby, strengthen disease surveillance and control strategies) in Egypt.

Finally, a cross-sectional study of *M. bovis* prevalence in the north-western Sokoto and Kebbi states of Nigeria reported 81% seropositivity among cattle herds (Tambuwal et al., 2017), while only ~20% prevalence was reported in the north-eastern Adamawa state (Francis et al., 2015b).

Asia, the Middle East and Oceania

As in Africa, excepting the recent emergence of *M. bovis* in New Zealand, relatively few studies over the past decade have focused on *M. bovis* epidemiology in Asia, the Middle East and Oceania. However, the reported prevalence of *M. bovis* is very high in Vietnam (Nguyen and Truong, 2015), with lower but still economically significant levels in Japan (Murai and Higuchi, 2019) and China (FuRong et al., 2012).

A few illustrative studies have emerged from the Eastern Mediterranean and the Middle East: in Israel, *M. bovis*-associated cases of mastitis were reported only rarely prior to 2008, when a sudden jump in prevalence led to ongoing annual infections of around nine herds per year in the country (Lysnyansky et al., 2017, 2016; Yair et al., 2020). Seroprevalence studies have also confirmed high levels of circulating *M. bovis* in Afghanistan (Bahir et al., 2017) and Pakistan (Ahmad et al., 2014; Mahmood et al., 2017).

Meanwhile, in Australia, the seroprevalence of *M. bovis* among feeder cattle was reported as 3.5% on feedlot induction and 25.3% six weeks later, indicating a high level of inter-farm transmission in this environment (Schibrowski et al., 2018b). Similar results were observed on Canadian feedlots and in another study of Australian feedlots, altogether suggesting that most *M. bovis* infections occur after feedlot entry rather than before (Barnewall et al., 2022; Castillo-Alcala et al., 2012). Studies of *M. bovis* among live export cattle leaving Australia have also observed a significant increase in prevalence between depot entry and resampling, emphasising the aforementioned role of transport in facilitating disease transmission (Moore et al., 2015, 2014).

Surveillance, transmission, and risk factors

Within herds, *M. bovis* is primarily spread via direct physical contact, through aerosols, and through contaminated milk (Calcutt et al., 2018; Castillo-Alcala et al., 2012; Kanci et al., 2017; Maunsell et al., 2011), and the introduction of subclinically infected replacement animals is a common source of infection in naïve herds ((Hazelton et al., 2018b; Pardon et al., 2020; Woolums et al., 2014); reviewed in (Fox et al., 2005)). Transmission via environmental contamination (e.g., of cattle pens, bedding, etc.) is thought to be negligible because of the generally poor environmental survival of mycoplasmas, but instances of putative indirect transmission have been reported (Justice-Allen et al., 2010; Piccinini et al., 2015), with the formation of a protective biofilm potentially allowing the fragile mycoplasma cells to survive on surfaces for long periods of time. Other, less common, avenues of transmission have

been hypothesised, and recent studies have begun to shed light on these. Following the detection of *M. bovis* in Finland, Haapala *et al.* published the first report of its introduction into a naïve cattle herd via processed semen from an infected bull, raising questions about the antibiotics used to keep these samples contamination-free during transport (Haapala et al., 2018). *M. bovis* DNA has also been identified at low frequency in colostrum, but further studies are needed to determine whether this colostrum is actually infectious (Gille et al., 2020; Timonen et al., 2020).

While research into these understudied means of transmission is ongoing, other studies have focused on direct contact transmission and the factors that affect its occurrence. The conditions of cattle transport, which is extremely common in the farming of most livestock, have been linked to the incidence of BRD in transported animals (reviewed in (Cusack, 2023)) – indeed, *M. bovis* positivity was found to increase ten-fold after travelling in a study of cattle transported from France to Italy, and stocking density and arrival temperature were also significantly correlated with the occurrence of *M. bovis* (Padalino et al., 2021). Several studies have confirmed that *M. bovis* prevalence among transported cattle is significantly higher on arrival (after transport) compared to loading (before transport) (Cirone et al., 2019; Stroebel et al., 2018).

Transport is one unavoidable factor in cattle farming that can substantially impact the risk of *M. bovis* infections – feeding is another. On dairy farms, automatic milk feeders were recently found to increase the risk of *M. bovis* transmission compared to bucket feeding, potentially via the formation of biofilms on shared nipples (Arcangioli et al., 2021). This study's authors also showed that the use of automatic milk feeders was associated with increased consumption of antibiotics, of which *M. bovis* is one of the primary drivers in cattle (Arcangioli et al., 2021; Bokma et al., 2020d). The development of drug resistance is therefore a major concern in the control of this pathogen. A longitudinal study of respiratory pathogens in Canadian beef calves reported high levels of resistance to macrolides including tulathromycin (Nobrega et al., 2021), and a report on antimicrobial resistance in bison isolates also showed decreased susceptibility to tulathromycin (Suleman et al., 2016b). Efforts to reduce antimicrobial use have met resistance from farmers due to concerns that lower treatment levels will lead to higher cattle mortality rates – however, a recent retrospective study of Belgian veal farms was unable to establish a negative correlation between antimicrobial use and mortality in that setting (Bokma et al., 2020a). With drug resistance receiving increasing attention from policymakers and the public over the past decade, further studies of the economic and herd-level effects of reducing antimicrobial use would be useful for establishing, for instance, the level of financial incentive necessary to promote high levels of compliance with drug resistance reduction initiatives.

Wildlife

The trajectory of *M. bovis* in American wildlife highlights the complex epidemiology of this pathogen outside cattle farms. Despite being present in American cattle since at least the 1960s, *M. bovis* was first observed to infect North American bison only decades later, with outbreaks causing up to 30% mortality in affected herds in Canada and the western USA (Register et al., 2021b). However, retrospective analysis of *M. bovis* seropositivity in North American bison revealed that these animals likely harboured subclinical infections since at least the late 1980s, indicating that the later emergence of clinical disease may be attributable to the evolution of new *M. bovis* genotypes (Register et al., 2021b). *M. bovis* is an emerging pathogen of farmed bison particularly in western Canada (Bras et al., 2016, 2017a), where it has been reported as the most common cause of death on bison farms (Epp et al., 2018), but its epidemiology in the broader geographical region remains poorly understood (Register et al., 2021a). Subclinical infections and maintenance of *M. bovis* in the upper respiratory tract appear to be common in bison, making it very difficult to gauge its true prevalence and transmission patterns among bison herds without targeted, active surveillance (Bras et al., 2017b; Register et al., 2021a). Acute *M. bovis* infections have also been identified in American pronghorn (*Antilocapra americana*), with environmental transmission from cattle considered the most likely vector for introduction (Johnson et al., 2022; Malmberg et al., 2020).

Control of the disease

Although *M. bovis* is pervasive and not subject to WOAH regulations (Dudek et al., 2020), it is one of the most economically burdensome mycoplasmas of livestock today. Effective approaches to its control have been made via government sponsored eradication programmes and voluntary control programmes paid for by the industry and farmers. Key to the control of *M. bovis* is regular surveillance, using accurate testing procedures and methods (Laven, 2019; Pohjanvirta et al., 2021) as asymptomatic carriers are common, and shedding of pathogen is irregular. The testing of individual animals or herds at a single time point is not reliable and it is thus key for accurate measurement of the infection status of herds, that sequential testing is carried out (Vähänikkilä et al., 2019).

To achieve *M. bovis* eradiation, the most effective control strategy is to cull animals with Mycoplasma disease due to *M. bovis* as current treatment options do not eliminate the pathogen. It is also noted

that antimicrobial resistance is developing with *M. bovis* from different geographical sources exhibiting high macrolide minimum inhibitory concentrations in many studies (reviewed in (EFSA Panel on AHAW et al., 2021). Coupled with this challenge, no independently validated clinical breakpoints for antimicrobials have been specified for *M. bovis*, making data interpretation and correlation of *in vitro* minimum inhibitory concentrations to *in vivo* efficacy in disease treatment difficult (Jelinski et al., 2020; Klein et al., 2017). Biosecurity measures that include surveillance of clinical symptoms, regular testing of bulk milk, animal trade control and the separation of infected cows remain effective control measures for *M. bovis*.

Policy

Approaches for controlling *M. bovis* range from government-funded control programmes based on surveillance/herd serology and eradication via culling, such as adopted in New Zealand (Jaye et al., 2022; Laven, 2019), to privately funded industry control based on voluntary reporting and control, as in Finland (Autio et al., 2021). Any approach taken relies on adequate and accurate testing, as asymptomatic carriers are common (Pohjanvirta et al., 2021); in the 2017 outbreak in New Zealand, 98% of *M. bovis*-positive test results were from animals with no clinical signs of disease (Laven, 2019). Within dairy herds, the cornerstone of detection of *M. bovis* is via bulk milk testing (Autio et al., 2021; Nicholas et al., 2016), with recent data suggesting that the preservation of *M. bovis* in milk for testing via culture or molecular methods at diagnostic facilities can be best achieved through the addition of a combination of glycine and dimethyl sulphoxide (DMSO) as a cryoprotectant prior for storage at -20°C or -80 °C (Al-Farha et al., 2018a).

The voluntary control programme in Finland has been in place since 2013, and the key elements are observation of clinical signs, nasal swab sampling from calves, testing bulk tank milk and clinical mastitis samples for *M. bovis,* and control of animal trade (Autio et al., 2021). Animal trade control involves the requirement of health certificates when purchasing cattle, where the purchase of cattle is only allowed from farms at the same or a higher level of biosecurity (Autio et al., 2021). Since *M. bovis* is largely untreatable, infection control advice is to cull cows with *M. bovis* mastitis and isolate their calves or prevent nose-to-nose contact with older animals for at least six months. Nicholas *et al.* weighed the pros and cons of culling and proposed an alternative control strategy to test-and-slaughter, based on regular testing, early diagnosis and separation of affected cows (Nicholas et al., 2016). Essentially, their eight recommendations were: weekly testing of bulk tank milk, milking hygiene measures, testing of milk samples from all cows before they enter or re-enter the lactating

herd, rapid segregation and contact monitoring of infected cows, judicious use of antibiotic treatments, and pasteurisation or disposal of waste milk, with culling recommended only when welfare is compromised (Nicholas et al., 2016).

Control

Pasteurising waste milk before feeding to calves is not possible in all situations, and disposal of milk can be undesirable. Beidel *et al.* demonstrated that the addition of dilute formic acid to *M. bovis*-infected raw milk to a final pH of ≤ 4.5 effectively kills the pathogen (Beidel et al., 2016). Similarly, milk acidification using Salstop, a commercially available feed acidification agent, was also effective at eliminating viable *M. bovis* (Parker et al., 2016a). The implementation of this management practice of acidification of waste milk would limit the transmission of *M. bovis* to susceptible calf populations where pasteurisation or UV-irradiation are not available.

The purchase of asymptomatic carrier animals that may harbour *M. bovis* in the airways or mammary glands is a major risk for its introduction into naïve herds; nasopharyngeal swabs or bronchoalveolar lavage sampling of calves for PCR detection, even if no *M. bovis* mastitis has been detected in the herd, has been recommended to manage this route of introduction (Pohjanvirta et al., 2021). Hazelton *et al.* cautioned that herd biosecurity protocols and control programmes should also account for the role of bulls used in breeding across herds in the introduction and spread of Mycoplasma species (Hazelton et al., 2018a). These authors monitored mycoplasma infection status of bulls pre- and post-breeding in four herds using culture and PCR; *M. bovis* was not isolated from any of the 150 bulls pre-breeding, but its seroprevalence increased from 9% pre-breeding to 46% post-breeding with no evidence of clinical disease, indicating that infected bulls could increase the risk of entry of *M. bovis* into other herds or, potentially, transmission within a herd (Hazelton et al., 2018a).

Similarly, Haapala *et al.* reported the introduction of *M. bovis* to two separate, biosecure dairy herds in Finland via artificial insemination from an *M. bovis*-positive bull, and they thus recommended the re-evaluation of antibiotics used in semen extenders or the provision of tested *M. bovis*-free semen for artificial insemination (Haapala et al., 2018). Notably, some of the most common antimicrobials added to semen extenders (e.g., penicillin, gentamicin, streptomycin, tylosin, spectinomycin and lincomycin) are not always effective in controlling mycoplasmas (García-Galán et al., 2020a). Other antimicrobials such as enrofloxacin or doxycycline were shown to efficiently inhibit the growth of *M. bovis* in several *in vitro* studies, but their efficacy had not been assessed in bovine semen. To support

new strategies for reducing the risk of *M. bovis* transmission through artificial insemination, Garcia-Galán *et al.* assessed the viability of *M. bovis* in bull semen diluted in a Tris-citrate-fructose solution after the addition of enrofloxacin, doxycycline or a *Lactobacillus*-based probiotic, reporting that all three were effective at reducing *M. bovis* viability (García-Galán et al., 2020a). Addition of *Lactobacillus* probiotic to cervical mucus *in vitro* also limited mycoplasma viability (A. García-Galán et al., 2020). The authors proposed that this may be due to a reduction in pH, and that the administration of *Lactobacillus*-based probiotics might be used in the future to control *M. bovis* proliferation in the cervico-vaginal tract of cows.

Sanitization of the farm environment and equipment where *M. bovis* is detected is important for biosecurity and preventing the spread of this pathogen. Mycoplasmas lack a cell wall and can be susceptible to osmotic lysis, although the presence of organic material (as expected to be found in agricultural environments) may protect them from lysis. Mahdizadeh *et al.* demonstrated that 0.5% citric acid and 1% sodium hypochlorite were effective disinfectants against *M. bovis* under field conditions in the presence of organic matter, while in the absence of organic material, 0.25% citric acid and 0.04% sodium hypochlorite were sufficient to disinfect against *M. bovis* (Mahdizadeh et al., 2020b).

Therapeutics

Antimicrobials used for the treatment or prevention of BRD usually include broad-spectrum cephalosporins (cefquinome and ceftiofur), extended-spectrum fluoroquinolones (enrofloxacin, danofloxacin, and marbofloxacin), florfenicol, and long-lasting macrolides (tulathromycin, gamithromycin, and tildipirosin). *M. bovis* from different sources has been reported to exhibit high macrolide minimum inhibitory concentrations in many studies ((Bokma et al., 2020b; Jelinski et al., 2020); reviewed in (EFSA Panel on AHAW et al., 2021)), indicating the development of resistance. Reported variabilities in strain susceptibility to antimicrobials may be related to geographical origin, year of isolation, type of livestock production system, clinical presentation, or site of isolation (reviewed in (Lysnyansky and Ayling, 2016)). In response to a mandate from the European Commission to investigate the global situation on resistant animal pathogens, the European Food Safety Authority (EFSA) reviewed studies from Asia, Europe and North America on the antimicrobial sensitivity of *M. bovis*; this analysis found relatively low mean levels of resistance to florfenicol and fluoroquinolones across the continents, whereas resistance to both macrolides and tetracyclines was much more pronounced (EFSA Panel on AHAW et al., 2021). Notably, the assessment of antimicrobial resistance

in *M. bovis* is circumscribed by the lack of approved interpretative criteria and standard procedures for susceptibility testing of mycoplasmas, leading to large uncertainty (EFSA Panel on AHAW et al., 2021).

Sulyok *et al.* determined the minimum inhibitory concentration (MIC) values of 35 *M. bovis* strains sampled in Hungary and reported that the most effective antibiotics tested *in vitro* were fluoroquinolones, though three fluoroquinolone-resistant isolates were identified; they also confirmed the reported increasing MIC values to antibiotics commonly used in the therapy of mycoplasma infections, primarily tetracyclines and macrolides (Sulyok et al., 2014a). The finding of fluoroquinolone-resistant isolates in Hungary and Great Britain (Ayling et al., 2014) is concerning, as many studies have found fluoroquinolones to be the most efficacious in inhibiting *M. bovis* growth and highlight a possible link between antimicrobial treatments and development of resistance in the *M. bovis* population (Barberio et al., 2016; EFSA Panel on AHAW et al., 2021; Heuvelink et al., 2016). Noting that standardised laboratory methods and interpretive criteria for MIC testing of veterinary mycoplasmas are needed, Klein *et al.* made use of a single laboratory to perform all of the antimicrobial resistance tests for 156 *M. bovis* isolates from France, Hungary, Spain, and the UK to ensure consistency in MIC values obtained (Klein et al., 2017). A similar strategy was used in a later study showing a moderate overall MIC_{50} increase of at most one doubling dilution for enrofloxacin, spiramycin, tylosin, florfenicol and oxytetracycline, and a contrasting reduction in the MIC_{90} value for oxytetracycline (Klein et al., 2019).

Studies in China and Japan note similar trends: *M. bovis* isolates from bovine respiratory infection outbreaks at beef farms in China were susceptible or had medium sensitivity to ciprofloxacin, enrofloxacin and doxycycline, but were frequently resistant to macrolides (Kong et al., 2016), while in Japan, *M. bovis* isolated from cases of bovine mastitis were sensitive to pirlimycin, danofloxacin and enrofloxacin, but not kanamycin, oxytetracycline, tilmicosin or tylosin (Kawai et al., 2014).

Many studies from around the world have investigated the antimicrobial sensitivity of *M. bovis* isolates from cattle, and these efforts have also recently been extended to isolates from bison. Tetracyclines, fluoroquinolones, and florfenicol failed to inhibit growth of bison isolates from Canada, demonstrating a marked difference from previously reported and laboratory reference cattle isolates (Suleman et al., 2016a). A study of clinical isolates from dead versus healthy cattle in western Canada indicated that those from dead cattle were more likely to be resistant to tulathromycin, gamithromycin, tylosin and enrofloxacin (Jelinski et al., 2020). These authors also noted high levels of antimicrobial resistance to

macrolides in all of the isolates. In a separate study in Alberta, Canada, Anholt *et al.* describe a high frequency of resistance to Category II antimicrobials (high importance to human health—neomycin, tylosin, tulathromycin, tilmicosin, and clindamycin), with extreme multidrug resistance in 30.5% of *M. bovis* isolates (Anholt et al., 2017). The differences between cattle and bison M. bovis isolates' antimicrobial sensitivity may be due to differences in management and paraphylaxis in the herds.

Understanding the evolution of *M. bovis* in susceptibility to the main classes of antimicrobials used to treat BRDs is useful and informative for future management strategies. A comparison of strains isolated 30 years apart (1978-1979 and 2010-2012) was carried out to assess the prevalence of acquired resistances on a national level in France (Gautier-Bouchardon et al., 2014). Only *M. bovis* isolated from BRD in young cattle were selected. Resistance to eight antimicrobials was shown to have been acquired over the 30-year period by all the strains, with particularly substantial increases in MIC_{50} values for tylosin, tilmicosin, tulathromycin and spectinomycin. The authors note that the first-line treatments currently recommended for BRDs in France target only *Pasteurellaceae*, which may promote mycoplasmosis and lead to chronic disease (Gautier-Bouchardon et al., 2014). A similar study of antimicrobial susceptibility profiles of *M. bovis* isolates collected from 1978 to 2009 in Ontario, Canada, was carried out to evaluate changes in MIC (Cai et al., 2019). Significant differences in MIC values across time were observed for chlortetracycline, oxytetracycline, tilmicosin, tylosin, clindamycin, tulathromycin, spectinomycin, danofloxacin, and gentamycin (Cai et al., 2019).

To use antimicrobial agents prudently and specifically in the treatment of *M. bovis* infections, targeted susceptibility testing should be performed prior to application of antimicrobial agents. For example, i*n vitro* antimicrobial sensitivity testing of *M. bovis* isolated from two flocks of sheep in India indicated resistance to the most-used antibiotics, but sensitivity to tylosin and enrofloxacin (Kumar et al., 2012). Treatment with tylosin was effective in promoting recovery within 15 days without recurrence (Kumar et al., 2012). However, conventional identification and antimicrobial susceptibility testing takes approximately two weeks for mycoplasma species. Additionally, there are currently no independently validated clinical breakpoints for antimicrobials specified for veterinary mycoplasma species, which makes data interpretation and correlation to *in vivo* efficacy difficult. Several research groups have demonstrated that high MIC values for *M. bovis* are associated with mutations in genes associated with antimicrobial resistance in other bacteria (Sulyok et al., 2017). Resistance to fluoroquinolones in *M. bovis* due to mutations in the *gyrA* and *parC* genes has been reported, for instance (Khalil et al., 2016; Sato et al., 2013; Sulyok et al., 2017). Lerner *et al.* observed that point mutations in the *M. bovis* 23S rRNA alleles were associated with decreased susceptibility to the macrolides tylosin and tilmicosin

(Lerner et al., 2014). Similar results were found in *M. bovis* isolates obtained from Japanese dairy calves (Sato et al., 2017) and confirmed via whole genome sequencing of Canadian isolates (Kinnear et al., 2020). Amram *et al.* investigated the mechanisms associated with acquired resistance to tetracyclines in *M. bovis* isolates from Israel, the United Kingdom, Germany, Spain, Australia, Hungary, Lithuania, and Cuba; they reported that mutations in the Tet-1 site in the *rrs* alleles of clinical *M. bovis* isolates were associated with decreased susceptibility to tetracycline (Amram et al., 2015). Resistance to tetracycline and spectinomycin is also associated with point mutations in the 16S rRNA gene altering the tetracycline or spectinomycin binding sites (Amram et al., 2015; Khalil et al., 2017; Sulyok et al., 2017). These combined findings indicate that genotypic approaches to assess antimicrobial susceptibility in *M. bovis* could be more useful as a standardised approach than culture-based techniques, as they would not be susceptible to variable results due to growth conditions.

The development of feasible diagnostic methods for the identification of *M. bovis* mutants is necessary to improve accessibility to appropriate antimicrobial therapy. A genome-wide association study to identify genetic markers linked to antimicrobial resistance in *M. bovis* was performed to link genotypes with phenotypes based on epidemiological cut-off thresholds (Bokma et al., 2021a). The authors assert that this approach shortens the present sample-to-result workflow to identify strains with genetic markers associated with acquired fluoroquinolone and macrolide resistance in a rapid and objective way, aiding decision-making in treatment. Mismatch amplification mutation assays and seven high resolution melt tests were designed to enable the rapid and cost-effective simultaneous detection of genetic markers and hot-spot regions related to increased MICs to antibiotics in *M. bovis* (Sulyok et al., 2018).

The efficacy of *M. bovis* treatments involving the combination of antimicrobials with or without other drugs has been tested. A preliminary study evaluated the effectiveness in calves of enrofloxacin given alone; in combination with flunixin meglumine, a nonsteroidal anti-inflammatory drug; or with an additional treatment of pegbovigrastim, an immunostimulatory (Dudek et al., 2019). Enrofloxacin given alone appeared to be the most effective treatment of the *M. bovis* affected calves in this case. A single dose of a combination of florfenicol-flunixin formulation administered subcutaneously to calves showing severe signs of respiratory disease was more effective in alleviating the clinical signs of disease than either florfenicol + flunixin meglumine or florfenicol alone (Thiry et al., 2014).

Combining antibiotics with nanoparticles was tested in a small-scale experiment to treat *M. bovis*-induced rabbit mastitis (Fathi et al., 2019). The authors tested a lincospectin + zinc oxide nanoparticle

and found it to be more effective than lincospectin alone, suggesting that antibiotic-tagged nanoparticles may increase the concentration of antibiotics at the site of bacterium-antibiotic interaction and thus improve their efficacy (Fathi et al., 2019).

Other potential alternative treatments in development include plant-derived antimicrobials such as food-grade trans-cinnamaldehyde, eugenol, and carvacrol which can inhibit the growth of *M. bovis in vitro*. Ranjitkar *et al.* investigated the mechanism of action of carvacrol and found that it affected the expression of 153 genes, including the downregulation of energy generation-related proteins and the pentose phosphate pathway and upregulation of ribosomes and translation-related proteins in *M. bovis* (Ranjitkar et al., 2022). The pathways affected indicate that carvacrol is a plant-derived antimicrobial worthy of further investigation.

Vaccines

In most of the world, there are not any licensed vaccines to prevent the suite of disease symptoms caused by infection with *M. bovis*, most likely because the use of whole-inactivated mycoplasma renders DIVA impossible by conventional tuberculin testing. The notable exception is the USA, where two bacterin vaccines targeting *M. bovis* are available: Myco-B contains a blend of three inactivated field isolates and their soluble antigens, while MpB Guard comprises a mixture of antigens from two field isolates of *M. bovis* and their soluble antigens (reviewed in (Dudek et al., 2021; Perez-Casal et al., 2017)). Previous studies suggest sub-optimal performance of these vaccines in the field, with significant protection observed in less than half of vaccinated calves (Soehnlen et al., 2011), and the risk of adverse reactions in young calves (Maunsell et al., 2009). In 2022, Protivity, a live-attenuated *M. bovis* vaccine, was brought to market in the USA by Zoetis, with the manufacturer reporting a 74% reduction in lung lesions of twice-vaccinated animals compared to unvaccinated control animals (USDA, 2022). However, this comes with caveats: the vaccine strain may also migrate to the joints and cause arthritis, and the duration of immunity and efficacy under field conditions are unknown; moreover, how the vaccine affects diagnostic testing for *M. bovis* is unclear.

Novel vaccine candidates continue to emerge from research groups around the world but have yet to be developed to commercial availability. A major challenge in eliciting effective immune responses is the high level of antigenic diversity of *M. bovis* variable surface proteins (see Immune evasion). Given the importance of DIVA, improved vaccines should also prioritise this feature in their design.

Novel vaccines

Various strategies have been trialled to generate potential novel vaccine candidates, with most studies highlighting the challenges of inducing protective immunity to experimental challenge with *M. bovis*. The use of an attenuated bacterial vector has shown the most promise among recently attempted strategies.

Inactivated vaccines

Building on earlier work by Nicholas *et al.* (Nicholas et al., 2002), Dudek *et al.* conducted a series of immunological studies characterising the antibody (Dudek and Bednarek, 2017a), cell-mediated adaptive (Dudek and Bednarek, 2017b) and acute phase (Dudek and Bednarek, 2018) responses to an inactivated *M. bovis* vaccine formulated with saponin (which acted both as the inactivator and an adjuvant) and lysozyme dimers. The vaccine induced a gradual increase in total immunoglobulins, with some signs of lymphocyte proliferation and acute-phase response activation, but protection from challenge was not reported in any of the studies.

The same group then characterised the bovine immune response to saponin-inactivated *M. bovis* either alone or in combination with other adjuvants (Dudek et al., 2018). They selected a combination of *M. bovis,* saponin and Emulsigen®, a commercially available adjuvant, and assessed its ability to protect calves from intratracheal challenge three weeks after a single immunisation (Dudek and Bednarek, 2018). The vaccine induced both mucosal and systemic antibody responses, significantly reduced disease severity and enabled almost all of the calves to clear *M. bovis* from their respiratory tract (Dudek and Bednarek, 2018), thereby representing a potentially promising candidate vaccine warranting further investigation in larger trials.

Live-attenuated vaccines

Zhang *et al.* immunised calves intranasally with two passage-attenuated *M. bovis* strains and challenged them intratracheally 46 days later (Zhang et al., 2014). Both attenuated strains induced *M. bovis*-specific IgG production and induced IFNβ production that was detectable in serum; vaccinated animals exhibited significantly milder clinical signs after challenge and shed significantly less *M. bovis* than unvaccinated controls, with the two strains eliciting approximately 70 and 80% protection overall (Zhang et al., 2014).

While intranasal immunisation is desirable for practical and potentially also immunological reasons, a recent study highlighted the need for caution: Bassel *et al.* reported an attempt to reduce the

susceptibility of newly arrived feedlot cattle to BRD by aerosol administration of a crude bacterial lysate of *Staphylococcus aureus* and *Escherichia coli*; they found that the inflammation induced by the lysate at the point of exposure to BRD-related pathogens, instead of protecting from these agents, in fact exacerbated disease susceptibility (Bassel et al., 2021). These findings highlight the need to investigate the optimal pre-exposure vaccination point in such settings, especially for mucosal vaccines, and also point towards a potential accessory role of pre-existing respiratory inflammation in enabling BRD.

Although live attenuated vaccines may induce high levels of immunity to a broad range of potentially protective antigens, they can also cause mild disease symptoms if incompletely attenuated. Before such vaccines could be widely adopted in the field, end-users would need to be aware of the risk of not being able to discriminate between infection with the pathogen and post-vaccine mild illness in the few days following immunisation; treatment with antibiotics at this stage would render vaccines ineffective, and this additional factor should be borne in mind during their development.

Subunit vaccines

Attempts to exploit *M. bovis* by immunisation with either total extracts or membrane fractions (Mulongo et al., 2013b), or with recombinant *M. bovis* glyceraldehyde 3-phosphate dehydrogenase (Prysliak et al., 2013), successfully elicited humoral responses but did not significantly protect from disease. Therefore, these researchers asked whether responses could be strengthened by using a cocktail of *M. bovis* proteins formulated with a combination of adjuvants. Again, despite earlier evidence of immune stimulation by the novel vaccine (Prysliak and Perez-Casal, 2016), this candidate formulation also failed to protect calves from virulent challenge (Prysliak et al., 2017).

Vectored vaccines

Briggs *et al.* generated and tested an attenuated *Mannheimia haemolytica*-vectored *M. bovis* vaccine expressing the cross-strain-conserved Elongation factor Tu and heat-shock protein-70; following a single immunisation, calves were dual-challenged with bovine herpesvirus 1 (to predispose calves towards disease) followed by *M. bovis* and assessed for mycoplasmal load and disease severity (Briggs et al., 2021). While the lungs of control animals exhibited high levels of *M. bovis*, the pathogen could not be isolated from the lungs of two thirds of vaccinated calves, and at a significantly lower level than controls in the remaining animals; accordingly, there were signs of mild disease only in a minority of the vaccinated group (Briggs et al., 2021).

Adjuvants

There have not been any notable advances published in this area since 2012.

Conclusions

M. bovis is responsible for animal mortality, lost productivity, and economic insecurity on cattle farms around the world, and with the rising prevalence of antimicrobial resistance, our options for controlling this pathogen are limited. Many *M. bovis* gene products remain uncharacterised, our understanding of basic aspects of host-pathogen interactions (e.g., the mechanisms underlying the establishment of chronic infection) is limited, and many *in vitro* findings remain to be validated *in vivo*. Still, the studies highlighted above give cause for optimism; great strides have been made in understanding the fundamental biology and epidemiology of *M. bovis*, its interactions with host animals, and the mechanisms underlying its infectivity. Alongside, new diagnostic assays could bring rapid, cost-effective pathogen detection even to remote locations, generating invaluable data on *M. bovis* transmission patterns and allowing more targeted surveillance and control programmes. Promising data from recent vaccine generation studies also open new avenues for controlling *M. bovis* on a large scale. With most of the world still lacking commercial vaccines for *M. bovis* (and therefore relying on antimicrobials to treat diseased animals), bringing an effective new vaccine to market could also reduce antimicrobial usage on many livestock farms, reducing the threat posed by drug resistance.

Future research priorities

Based on the available literature, expert opinion, and previously published reviews (Calcutt et al., 2018), we suggest that the following areas of research into *M. bovis* should be considered high priority:

Biology of the pathogen

- *Ongoing characterisation of* M. bovis *gene functions, including the use of modern gene editing techniques e.g. CRISPR/Cas to validate*
- *Definition of nutrient requirements in the different host tissues*
- *Comparison of field isolates' virulence and correlation with genomic/proteomic data*
- *Relevance of biofilms, and definition of their on-farm properties*
- *Assessment of the biological significance of intracellular infection*

Diagnosis

- *Standardisation of diagnostic protocols*
- *Development of a cost-effective pen-side test*
- *Antimicrobial sensitivity testing*

Pathogenesis

- *Understanding the factors driving the transition from asymptomatic infection to pathologic* M. bovis*-associated respiratory disease*
- *Adoption of standardised aerosol challenge method for calves, and design and validation of a similar system suitable for use with adult cattle*
- *Characterisation of host- and pathogen-associated factors underpinning highly pathogenic* M. bovis *infections of bison*
- In vivo *validation of putative virulence factors identified by genome sequencing and targeted mutation studies*
- *Development of a reproducible intramammary challenge model in small ruminants (or a better/easier/more affordable one in cattle) to allow studies of pathogenesis, local immunity and vaccine efficacy against* M. bovis *mastitis*
- *Comparative genomics of* M. bovis *isolated from different tissue sites to generate insight into tissue-specific adaptations/virulence factors*
- *Increased use of primary cells/tissue explants/field samples: data generated in cell lines are often contradictory and/or with unknown relevance to the* in vivo *situation*

Immunology

- *Immunogenetics of increased* M. bovis *resistance, with a focus on innate immune factors that could also protect against other members of the BRD complex*
- *Increased use of primary cells/tissue explants/field samples: data generated in cell lines are often contradictory and/or with unknown relevance to the* in vivo *situation*
- *Identification of humoral correlates of protection*
- *Understanding of immune factors underlying polarisation to maladaptive Th17/Th2 versus adaptive Th1 immunity in infected animals*
- *Studies of the potentially protective and/or vaccine-interfering role of maternal immunity*
- *Defining the role of the host immune response in lesion development*

Epidemiology

- *Studies on the economic impacts of reducing antimicrobial use*
- *Understanding the impact of subclinically infected carriers and those cattle with* M. bovis *arthritis only*
- *Determining the environmental survival/tenacity of* M. bovis *under different climatic conditions*
- *Defining the role of wildlife in* M. bovis *transmission*
- *Targeted active surveillance of* M. bovis *in North American bison populations*
- *Studies to define true prevalence in mastitis, respiratory disease and arthritis in various field settings*

Control

- *Given the importance of DIVA for current control strategies, all new vaccine candidates should be compatible with this aim; therefore, subunit or vectored vaccines are likely preferable*
- *Field testing of the Protivity vaccine under a range of conditions to understand its efficacy*
- *Identification on antigens common to a range of* M. bovis *strains to facilitate subunit/vectored vaccine development*
- *Modelling and field-testing of promising non-cull control programmes*
- *Assessment of novel plant-derived antimicrobials showing promise in preliminary tirals, such as carvacrol*

Other mycoplasmas affecting cattle

Introduction

While *M. bovis* is generally the most prevalent and widely researched pathogenic mycoplasma of cattle, other less common species are also associated with bovine disease. These include the respiratory pathogens *M. bovirhinis* and *M. dispar*; the reproductive pathogen *M. bovigenitalium*; and the haemotropic mycoplasmas (haemoplasmas), an understudied class of obligate erythrocyte-associated pathogens that cause infectious anaemia and can be transmitted by arthropod vectors. There is also some speculation among those working in the field that *M. arginini* could play an important role during *M. bovis* pneumonia, but this has yet to be formally established. The study of these pathogens is a much smaller field compared to the more economically significant veterinary mycoplasmas, but studies published since 2015 have delivered valuable new insights into their biology and epidemiology.

Literature review

Biology of the pathogens

Little has been published over the past decade on the molecular biology of the more minor mycoplasmas of cattle, but the generation of new complete sequence data is an important step for the identification of critical proteins, virulence factors, and immune evasion-related factors in future studies of their functionality.

Molecular typing and genome sequencing

A draft genome sequence of *M. bovigenitalium* was published in 2013 (Lucía Manso-Silván et al., 2013), and the complete genome sequence of the Japanese strain HAZ 596 was published four years later (Hata et al., 2017a).

Three complete *M. bovirhinis* genomes have recently been published: the type strain PG43, the Chinese isolate GS01 (S. Chen et al., 2018), and the Japanese isolate HAZ141_2 (Hata et al., 2017b). A GC-rich prophage-like region of the HAZ141_2 genome was found to contain an *aadE-sat4-aphA-3* gene cluster conferring resistance to kanamycin and neomycin (Lysnyansky and Borovok, 2021a),

though an attempt to characterise the impact of genomic rearrangement events within this cluster was inconclusive (Lysnyansky and Borovok, 2021b).

Chen *et al.* published the complete genome sequence of *M. dispar* strain GS01, annotating putative virulence genes and pathogenicity islands (Chen et al., 2019).

Finally, the draft genome sequence of the Mexican *M. wenyonii* strain INIFAP02 was published in 2018 (Quiroz-Castañeda et al., 2018). This strain was later characterised by Flores-García *et al.*, who explored the phylogenetics of 12 reported bovine haemoplasma genomes – including two from *M. wenyonii* and one from '*Candidatus* Mycoplasma haemobos' (*C*. M. haemobos) – to outline their main genomic characteristics and identify B cell epitopes in strain INIFAP02 (Flores-García et al., 2022).

Proteomics and gene characterisation

There have not been any notable advances published in this area since 2015.

Diagnosis

The clinical signs associated with infection by these cattle mycoplasmas can be significant but are often nonspecific, necessitating the development of diagnostic assays that can discriminate them from each other and from other common cattle pathogens (e.g., *M. bovis*).

DNA diagnostics

Though primarily associated with reproductive and respiratory infections respectively, *M. bovigenitalium* and *M. bovirhinis* have both been associated with economically significant cases of cattle mastitis (reviewed in (Parker et al., 2018)). Both are frequently included among the secondary targets for multiplex qPCR assays designed primarily to diagnose *M. bovis* infection, with other mycoplasmas detected via the conserved 16S rRNA gene or 16S-23S rRNA intergenic spacer region (Chauhan et al., 2021; Gioia et al., 2016; Lai et al., 2022; Parker et al., 2017b). High-resolution melting curve assay has been applied to distinguish *M. bovirhinis* from *M. bovis* and other pathogens present in milk (Al-Farha et al., 2018b).

As a cause of granular vulvovaginitis, *M. bovigenitalium* has also been discriminated from *M. bovis* and *Ureaplasma diversum* present in vaginal swabs via nested PCR, targeting partial 16S-23S rRNA intergenic spacer region fragments for species-specific amplification (Voltarelli et al., 2018).

M. dispar was one of a number of mycoplasmas detected in a combined DNA microarray and qPCR study of the respiratory tract of Italian dairy calves; the authors of this study optimised a previously published microarray assay targeting the mycoplasma 23S rRNA and *tuf* genes (Schnee et al., 2012) to identify >70 mycoplasmas and reported particularly high prevalence of *M. dispar* among tested animals (Bottinelli et al., 2017).

Freeman *et al.* used third-generation Oxford Nanopore metagenomic sequencing to detect *M. dispar* and other mycoplasmas in chronically infected feedlot cattle and to analyse their expression of antimicrobial resistance genes (Freeman et al., 2022). Nasal microbiome sampling has also been used to identify BRD complex mycoplasmas present in the upper respiratory tract, including *M. bovirhinis* and *M. bovis* (Centeno-Martinez et al., 2022).

Finally, Ade & Niethammer *et al.* developed qPCR assays specifically for *M. wenyonii* and *C.* M. haemobos by targeting their dehydrogenase-encoding *gapN* genes (Ade et al., 2018).

Serological diagnostics

A serodiagnostic assay has also been developed for *M. wenyonii*, with Zhao *et al.* using an antigen-specific colloidal gold immunochromatographic test to specifically detect antibodies against this pathogen in cattle serum samples (Y. Zhao et al., 2017b).

Sampling strategies and other developments

There have not been any notable advances published in this area since 2015.

Pathogenesis

Research on the pathogenesis of these cattle mycoplasmas has been limited over the past decade, but a small number of *in vivo* and *ex vivo* studies have been published on their host-pathogen interactions and associated clinical signs.

In vivo *and* ex vivo *studies*

Pôrto *et al.* exposed primary bovine epithelial cells from the vagina or endometrium, and PBMC, to *M. bovigenitalium ex vivo* and documented the production of nitric oxide after 48 hours, concluding that immune cells from these sites are potentially involved in the initial recognition of and the response to the pathogen (Pôrto et al., 2021).

A study in Brazil aimed to identify the pathogens present in a cohort of calves with signs of respiratory disease, and documented *M. dispar* in almost 40% of cases (de Oliveira et al., 2016). These data point to *M. dispar* as potentially having a more significant role in the pathogenesis of respiratory disease in this setting than previously appreciated.

Two case studies were recently published on novel presentations of *M. wenyonii*: one report of a dairy cow exhibiting haemolytic anaemia secondary to infection (Gladden, 2015); and another of exophthalmos of the eyes in an infected calf (Alsaad et al., 2021).

A single study reported the possibility of vertical transmission of *M. wenyonii* from infected dams to their neonatal offspring, in addition to the widely accepted arthropod-mediated route (Sasaoka et al., 2015). The authors observed a transmission rate of almost one quarter, in the absence of milk testing positive for the pathogen (Sasaoka et al., 2015).

In vitro *studies*

There have not been any notable advances published in this area since 2015.

Immunology

There have not been any notable advances published in this area since 2015.

Geographic distribution and epidemiology

The great majority of cattle mycoplasma epidemiology studies published over the past decade have focused on *M. bovis*, but several reports have emerged on the prevalence of the more minor mycoplasmas in disparate geographical regions.

Global situation

The Americas

M. bovigenitalium infections are associated with reproductive and respiratory pathologies, but its prevalence is generally far lower compared to *M. bovis* (Hashem et al., 2022b), and relatively few studies of its epidemiology have been conducted. Infection with *M. bovigenitalium* has been associated with reproductive disorders in Brazil, where it is widespread on beef and dairy cattle farms (Carli et al., 2022; Macêdo et al., 2018).

Haemoplasma infections are particularly widespread in cattle and water buffalo (*Bubalus bubalis*) in Brazil (Santos et al., 2018), though reported prevalence varies widely across the country; in the major dairy-producing municipality Ji-Paraná, herd-level prevalence of *C.* M. haemobos was estimated at >95% (Witter et al., 2017), while only ~2.3% was reported in the Pantanal region (Mello et al., 2019).

Europe

M. dispar is frequently detected alongside *M. bovis* and *M. bovirhinis* in the nasal cavity of beef and dairy cattle, and several studies over the past decade have reported its prevalence levels across Europe in particular (Antonis et al., 2022; Deeney et al., 2021; Gaeta et al., 2018; Szacawa et al., 2016, 2015). *M. dispar* is widely prevalent in Switzerland, for instance, though *Pasteurellaceae* species, *Mannheimia haemolytica* and *Histophilus somni* were more common respiratory pathogens (Schönecker et al., 2020). *M. dispar* and *bovirhinis* also appear to be widely prevalent in North American bison populations, where many animals may be asymptomatic carriers of these pathogens (Register et al., 2021a).

In France, cases of acute milk production loss, anaemia, and oedema led to the first detection of *M. wenyonii* infections in the country, with overall prevalence in Brittany estimated at ~26% (Nouvel et al., 2019). Hornok *et al.* assessed the prevalence of several vector-borne bacterial pathogens among large game animals in Hungary, finding >90% prevalence of *M. wenyonii* in water buffalo (Hornok et al., 2018). Meanwhile, in Bosnia and Herzegovina, Stevanović *et al.* reported the first detection in Europe of *M. wenyonii* DNA in castor bean ticks (*Ixodes ricinus*) feeding on infected cattle (Stevanović et al., 2020). Haemoplasma prevalence studies have also been conducted in Germany (Niethammer et al., 2018) and the United Kingdom (Deeney et al., 2021).

Africa

Boularias *et al.* reported the first detection of *M. wenyonii* and *C.* M. haemobos in northeast Algeria (Boularias et al., 2020) and Byamukama *et al.* conducted the first molecular detection and characterisation of haemoplasma infections in Ugandan cattle and goats, reporting ~32% and 44% positivity, respectively (Byamukama et al., 2020). Reported prevalence levels are higher in Somalia, where nearly 100% of cattle were haemoplasma-positive in a recent diagnostic study (Ferrari et al., 2022).

Asia, the Middle East and Oceania

In 2020, Mashhour *et al.* reported the first detection of *M. dispar* in Iran (Mashhour et al., 2020).

The prevalence of haemoplasmas including *M. wenyonii* has been investigated in several countries including Iraq (Jarad and Alsaad, 2016), Malaysia (Mohd Hasan et al., 2017; Ola-Fadunsin et al., 2017), the Philippines (Galon et al., 2020; Ybañez et al., 2019) and Japan (Tatsukawa et al., 2021).

Surveillance, transmission and risk factors

There have not been any notable advances published in this area since 2015.

Wildlife

There have not been any notable advances published in this area since 2015.

Control of the disease

As many of the cattle mycoplasmas are either commensals or commonly co-infect in *M. bovis*-infected cattle, control measures based on animal management and good biosecurity practices will also be effective in their control. Antimicrobial treatments should be judiciously and specifically applied in treating mycoplasma infections to avoid development of antimicrobial resistance, including in non-target organisms.

Policy

There have not been any notable advances published in this area since 2015.

Therapeutics

There have been few studies assessing the antibiotic sensitivity of *M. dispar* despite it being widely prevalent in the bovine population and exposed to untargeted chemotherapy for many years. Assays of the MIC of *M. dispar* isolates collected in Italy between 2011–2019 found a drift, similar to that seen in *M. bovis,* towards high antimicrobial concentrations, indicative of an ongoing selection process among the isolates (Bottinelli et al., 2020). The genetics or mechanisms of resistance in *M. dispar* were not investigated in the study. Testing antimicrobial susceptibility of bovine mycoplasmas by traditional methods such as broth microdilution is time-consuming because of the culture characteristics of mycoplasmas; thus, rapid detection of resistance-associated mutations would be beneficial in assessing the antimicrobial susceptibility of specific mycoplasma strains. Although rarely identified as a primary pathogen in diseases in many countries (Deeney et al., 2021), mastitis due to *M. californicum* has been on the rise recently in Japan (Hata et al., 2019). A genetic method involving melting curve analysis with a hybridization probe for the rapid detection of mutation loci associated with changes in antimicrobial susceptibility in *M. californicum* has been described (Hata et al., 2019). The authors were able to identify mutation points associated with susceptibility to macrolides and lincosamides within hours, meaning that the method has the potential to allow field veterinarians to make timely decisions about best treatment options.

As a result of the low immunogenicity of *M. dispar* in calves, the use of complex formulations that combine antimicrobials with prophylactic treatments that boost metabolism and immunity are recommended. The therapeutic efficacy of gentaminoseleferon (a combination therapy containing

gentamicin sulfate, aminoseleton, and a mixture of bovine recombinant IFN-α and -γ proteins) was compared with sulfetrisan (a mixture of sulphadimethoxine, erythromycin, and trimethoprim) in calves with respiratory infections that included *M. dispar* (Alhussen et al., 2020). The authors reported that the calves treated with gentaminoseleferon had a shorter recovery time and displayed reduced markers of inflammation in the blood and serum (Alhussen et al., 2020).

Vaccines

There have not been any notable advances published in this area since 2015.

Conclusions

Alongside *M. bovis*, these other mycoplasmas of cattle cause significant economic losses around the world, and their importance in individual infections and in co-infection with other cattle pathogens should not be neglected. The development of multi-target diagnostics (e.g., multiplex qPCR) primarily targeted at *M. bovis* has provided new opportunities for the simultaneous detection of *M. bovigenitalium*, *M. bovirhinis* and *M. dispar*, and emerging molecular technologies may also facilitate a greater range of data collection on the haemoplasmas ((Flores-García et al., 2022); reviewed in (Parker et al., 2018)). Future studies will be required to clarify the epidemiology of these pathogens (including the role of arthropod vectors in haemoplasma transmission in different climates and farm management systems), map their host-pathogen interactions (both alone and in co-infections), and monitor the development of antimicrobial resistance in these populations.

Future research priorities

Based on the available literature and expert opinion, we suggest that the following areas of research into other mycoplasmas affecting cattle should be considered high priority:

Biology of the pathogens

- *More complete sequences alongside functional characterisation of bovine mycoplasma genomes*

- *Comparison and molecular typing of genome sequences to support phylogenetic, evolutionary and epidemiological studies*

Diagnosis

- *Validation and deployment of diagnostic assays for bovine haemoplasmas*
- *Continuing focus on the development of field-capable assays useable in resource-limited settings*

Pathogenesis

- *Clarification of the respiratory pathogenicity of* M. bovigenitalium *alone or in co-infections with BRD complex-associated pathogens*
- *Understanding the potential roles of* M. bovigenitalium *and* M. bovirhinis *in clinical mastitis*
- *Continuing study of strain-specific virulence factors*
- *Studies of potential novel clinical presentations of bovine haemoplasma infection*

Immunology

- *Characterisation of host-pathogen interactions and mechanisms underlying immune evasion*

Epidemiology

- *Standardisation of bovine mycoplasma prevalence studies, including sampling methods, sample storage, diagnostic assays, and data interpretation*
- *Identification of region-specific arthropod vectors for bovine haemoplasmas*

Control

- *Continuing studies of antimicrobial sensitivity*
- *Studies of potential vaccine candidates*

Mycoplasmas affecting swine

Introduction

The four primary mycoplasmas that will be discussed in this section – *Mycoplasma flocculare* (*Mfl*), *M. hyopneumoniae* (*Mhp*), *M. hyorhinis* (*Mhr*), and *M. hyosynoviae* (*Mhs*) – are commonly found in the respiratory tracts of both healthy and diseased pigs (Ferrarini et al., 2016). *Mfl* is a commensal bacterium that is closely related to the pathogenic *Mhp* (Paes et al., 2017a; Sonalio et al., 2022), while *Mhr* and *Mhs* have been referred to as commensal and pathogenic in the literature (Ferrarini et al., 2016; Klose et al., 2022a; Roos et al., 2019; Wegner et al., 2020).

Mhp, the primary pathogen of swine enzootic pneumonia, is by far the most clinically significant of these four mycoplasmas. It is widespread across the globe and is one of the most common and economically important bacterial pathogens of the swine respiratory tract (Brewster et al., 2017; Pieters and Maes, 2019; Taylor, 2013). Enzootic pneumonia is associated with a dry, chronic cough and macroscopic cranioventral pulmonary consolidation lesions and, although rarely fatal, has been linked with lower average daily weight gain and feed conversion ratio (reviewed in (Bargen, 2004; Maes et al., 2008)). Additionally, while the economic burden imposed by uncomplicated *Mhp* infections is thought to be relatively small, such infections are rare (Hoist et al., 2015); more often, *Mhp* is found in co-infection with other swine respiratory pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza A virus (sIAV), *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae*, which can increase the severity of these infections and lead to higher mortality rates ((Fablet et al., 2016; Paiva et al., 2023; Tonni et al., 2022); reviewed in (Oba et al., 2020)). Co-infections between *Mhp* and PRRSV, for instance, were estimated to cost 9.69 USD (12.74 USD adjusted for inflation in 2023) per head in a study of a large-scale pig production system in the USA, and *Mhp*-sIAV co-infections were even costlier (Haden et al., 2012). Accordingly, the majority of this section is dedicated to a representative selection of the substantial number of research studies published on *Mhp* over the past decade.

Meanwhile, *Mhr* and *Mhs* are generally considered commensal microbes, but both have been associated with swine polyarthritis, and *Mhr* has recently been reported to contribute to mortality in nursery pigs (Clavijo et al., 2017, 2019b; Roos et al., 2019). *Mfl* has not been individually linked to any pathogenic activity, but it is present in pneumonia-like lesions and has been reported to potentially aggravate these lesions during *Mhp* infection (Ferreira et al., 2021; Fourour et al., 2019c).

Recent research on the swine haemoplasmas (haemotropic mycoplasmas) will also be addressed in this section. These uncultivable organisms comprise the pathogenic *M. suis*, the non-pathogenic *M. parvum*, and the recently discovered *Candidatus* Mycoplasma haemosuis (*C.* M. haemosuis). *M. suis* is the causative agent of infectious anaemia in pigs and can cause high mortality in piglets and feeder pigs in severe cases (Stadler et al., 2021). However, despite its high prevalence in many countries, the biological properties, epidemiology and economic impacts of *M. suis* remain poorly understood.

The studies cited below from 2015-23 have begun to close some important knowledge gaps, but much remains to be done before the threat posed by swine mycoplasmas has been fully neutralised.

Literature review

Biology of the pathogens

The fundamental biology of swine mycoplasmas is very similar to that of the other mycoplasmas discussed in this report: tiny obligate parasitic bacteria that lack a cell wall, are naturally resistant to many antibiotics, and are generally difficult to grow in culture. Molecular biology studies of these organisms published over the past decade have primarily fallen into three general categories: molecular typing, complete genome sequences, and genomic/proteomic studies.

Molecular typing and genome sequencing

The ability to differentiate co-circulating mycoplasma strains is critical for effective surveillance of the porcine respiratory microbiome. To address these needs, many MLST protocols have been published for swine mycoplasmas over the past decade. Tocqueville *et al.* developed two methods for *Mhr* differentiation – a highly sensitive TaqMan qPCR assay targeting the p37 gene and an MLST strategy based on six housekeeping gene fragments – and compared them on a representative set of 33 *Mhr* strains, reporting superior discriminative power by the MLST (Tocqueville et al., 2014). These authors also set up a database for this method as part of PubMLST. A later study used an optimised version of this method (with new primers allowing single-protocol amplification and sequencing) to assess *Mhr* diversity on Swiss and German pig farms, finding that *Mhr* had similar population structure to *Mhp*, with high variability and low clonality (Balestrin et al., 2019; Trüeb et al., 2016). More recently, Bünger *et al.* developed a core genome MLST (cgMLST) workflow for *Mhr* based on 453 target genes and

reported superior typing performance compared to traditional MLST and core genome SNP analysis (Bünger et al., 2021).

An MLVA protocol based on two hypothetical proteins of *Mhr* (MHR_0152 and _0298) was published in 2015 and was used to identify 16 MLVA types within 165 samples in the USA (Dos Santos et al., 2015a). This group next developed an *Mhr* MLST method that included both housekeeping and surface protein-encoding genes, as surface proteins generally face greater selection pressure and can thus be more useful for comparing closely related strains (Clavijo et al., 2019b). Later, Földi *et al.* combined MLVA and MLST for sequence typing of 40 *Mhr* strains from Central Europe, reporting that MLST produced a more robust phylogenetic tree while MLVA allowed differentiation of closely related isolates (Földi et al., 2020).

Following an early report that *Mhp* strains could be sufficiently discriminated by MLST based on just three highly variable targets (the *adk*, *rpoB* and *tpiA* genes) (Mayor et al., 2008), this strategy was applied to study strains circulating within several countries including China (H. Zhang et al., 2021) and Thailand (Tadee et al., 2018). MLVA has also been widely used to discriminate between closely related *Mhp* isolates (e.g., within single farms or individual pigs) (Dos Santos et al., 2015b; Pantoja et al., 2016; Rebaque et al., 2018; Sosa et al., 2019; Tonni et al., 2021) and between circulating field and vaccine strains (Tamiozzo et al., 2015), and efforts have been made to standardise the terminology and classification used for *Mhp* strains based on MLVA (reviewed in (Betlach et al., 2019)). Finally, Felde *et al.* compared four methods of *Mhp* typing (three-gene and seven-gene MLST systems, MLVA, and *p146* gene analysis), reporting that a combination of the minimal MLST, MLVA, and *p146* analysis was most effective sequentially for increasingly smaller-scale, higher-resolution discrimination (Felde et al., 2018b). These same three genes (*adk*, *rpoB* and *tpiA*) were targeted to develop an MLST scheme for *Mfl*, with the authors reporting high discriminatory power; this workflow was also made available on PubMLST (Fourour et al., 2019a).

Finally, the past ten years have seen the publication of a few new complete genomes for swine mycoplasmas, including *Mfl* strain Ms42^{T} (Calcutt et al., 2015), *Mhp* strain KM014 (Han et al., 2017), and *Mhr* strains SK76 (Goodison et al., 2013) and DSM 25591 (Käbisch et al., 2021). The first draft genome sequences of seven *Mhs* strains were published in 2014, alongside genomic analysis of putative virulence factors, CRISPR/Cas sites, and other regions of interest within the *Mhs* genome (Bumgardner et al., 2014, 2015).

Proteomics and gene characterisation

Characterisation of mycoplasma genomes, protein products, and the molecular mechanisms underlying transcription and translation is critical for understanding their virulence and pathogenic properties and for the development of effective vaccines. As the primary pathogen of swine enzootic pneumonia, *Mhp* has unsurprisingly been the focus of many of these studies over the past decade. Cattani *et al.* scanned the intergenic regions of all coding sequences in the *Mhp* strain 7448 genome, reporting a total of 1,315 palindromic elements and tandem repeats that may be involved in transcriptional regulation (Cattani et al., 2016), while Siqueira *et al.* identified 47 small RNAs in the genome of *Mhp* and 11 in *Mhr* (Siqueira et al., 2016); this group later conducted a focused analysis of the *Mhp* small RNAs and their associated target sites and regulatory elements, reporting differential expression of target genes in response to oxidative stress conditions in culture (Fritsch et al., 2018). Interestingly, a recent genomic and proteomic study of *Mhp* reported that the pathogen is missing several genes involved in formylated N-terminal methionine processing, indicating that *Mhp* has evolved a translation mechanism that does not require this standard initiator of protein synthesis (Jarocki et al., 2019b). Finally, in Tibet, a study of the local *Mhp* TB1 strain revealed mutations consistent with adaptation to the plateau environment (e.g., in hypoxia-associated genes) (Gang et al., 2019).

Zhu *et al.* reported their identification of the *Mhp* gene *Lpl*, which encodes a lipoate protein ligase that is involved in the generation of this critical cofactor for metabolic enzymes (K. Zhu et al., 2020). Ishag *et al.*, meanwhile, transformed *Mhp* and *Mhr* with plasmids encoding GFP driven by the *p97* gene promoter, showing that they could be used to monitor gene transfer and expression *in vitro* (Ishag et al., 2016b, 2016a). This group later developed *oriC*-plasmids and successfully transformed them into *Mhr*, demonstrating proof-of-concept by targeting the *hlyC* gene to produce hemolysin-mutant bacteria (Ishag et al., 2017).

Mycoplasma resistance to fluoroquinolones is often attributable to mutations in the target proteins of these drugs, DNA gyrase and topoisomerase IV (reviewed in (Gautier-Bouchardon, 2018)). Accordingly, a recent molecular investigation of drug resistance in Chinese *Mhr* isolates identified a total of ten point mutations in bacterial DNA gyrase (GyrA) and topoisomerase IV (ParC and ParE) proteins that were associated with reduced susceptibility to fluoroquinolones (J. Li et al., 2022b).

Finally, recent investigations of pathogenic and non-pathogenic *Mhp* strains have produced interesting results for future comparative studies. Garcia-Morante *et al.* reported identical growing phases and maximal titres during culture of the non-pathogenic type strain J and pathogenic 11 and 232 strains, though the J strain exhibited the fastest growth (Garcia-Morante et al., 2018). Meanwhile, a pathogenic and non-pathogenic strain of *Mhp* have both been shown to produce extracellular vesicles *in vitro* (de Souza et al., 2022); this published method for extracellular vesicle isolation will now permit future studies of vesicle composition/contents and their biological relevance.

Diagnosis

Mhp has dominated most research published on the diagnosis of swine mycoplasmas over the last decade, and many techniques have been used to detect *Mhp* in various sample types. Culturing is considered the gold standard, but like all mycoplasmas, *Mhp* is extraordinarily difficult to isolate; more practical methods include immunofluorescence, serology and PCR (Oh et al., 2020). Diagnosis of *Mhp* is made more challenging by its chronic nature and tropism for the lower respiratory tract (reviewed in (Garcia-Morante et al., 2022; Pepovich, 2020)) and the variable sensitivity of diagnostic assays over the course of infection (Sponheim et al., 2021). *Mhp*-induced lung lesions are an important part of the macroscopic detection of enzootic pneumonia – various scoring systems have been published for assessing and categorising these lesions, and methods have been developed for comparing the results of these different systems (reviewed in (Garcia-Morante et al., 2016)). Laboratory diagnostics are then required to positively identify the pathogen (Silva et al., 2022). As with other mycoplasmas, these assays are mainly divided into nucleic acid diagnostics and serodiagnostics; however, advances have also been made in sampling strategies and other diagnostic avenues, and these will be discussed below as well.

DNA diagnostics

The high frequency of co-infections in the swine respiratory tract has spurred the development of assays intended for the simultaneous detection and differentiation of individual pathogens. These include a multiplex TaqMan qPCR for distinguishing *Mhp*, *Mhr* and *Mfl* by targeting the *p102*, *p37* and *fruA* genes (Fourour et al., 2018); a duplex qPCR assay for discriminating *Mhp* and *Mhr* in several pig clinical sample types (YuZi et al., 2019); and multiplex qPCR assays for detecting *Mhp* and other swine respiratory pathogens in a single run (Rao et al., 2023; Sunaga et al., 2020). Lung *et al.* developed a

prototype automated microarray assay and associated multiplex PCRs for diagnosis of *Mhp* and seven other important viral and bacterial pathogens of swine (Lung et al., 2017). Meanwhile, as in their publication on *M. bovis*, Goecke *et al.* developed a protocol for evaluating enzootic pig pathogens (including *Mhp* and *Mhr*) on the high-throughput BioMark qPCR platform, reporting similar performance and substantial efficiency gains compared to traditional qPCR (Goecke et al., 2020). Digital PCR has also been applied for direct quantification of *Mhp* within bronchoalveolar lavage samples (Beuckelaere et al., 2022).

As mentioned in other sections, high-resolution melting curve analysis is another option for the differentiation of closely related pathogens from the same sample. One such assay was recently developed for both swine and bovine mycoplasma infections, distinguishing between *Mhp*, *Mhr* and *Mhs* cultured from swine lung samples while also detecting *M. bovis* from bovine lungs (Ahani Azari et al., 2020).

These studies have presented many new options for simultaneous diagnosis of multiple porcine respiratory pathogens; meanwhile, other groups have focused on the development of field-capable assays via isothermal molecular diagnostics that do not require a thermocycler. A LAMP procedure targeting the *p36* gene was developed for *Mhp*, and the authors reported 100% concordance with traditional qPCR when tested on lung tissue samples from experimentally infected swine (Liu et al., 2015). Meanwhile, combining an *mhp165*-targeted isothermal RPA assay with lateral flow strip detection provided 100% diagnostic specificity and ~89% sensitivity, requiring < 20 minutes for visual readout of test results (Liu et al., 2019).

Alongside, two studies specifically addressed the suitability of different sample types for PCR analysis, aiming to clarify which of the many sampling techniques currently in use may provide the best molecular diagnostic sensitivities. Pieters *et al.* compared five sample types (nasal and laryngeal swabs, oral and tracheobronchial lavage fluids, and blood) for PCR diagnosis of pigs experimentally infected with *Mhp*, finding that laryngeal swabs from 5+ days post-inoculation provided the highest sensitivity for DNA detection (Pieters et al., 2017). Meanwhile, Poeta Silva *et al.* recently compared two commercial DNA extraction methods and three PCR protocols for measuring within-pen *Mhp* prevalence from pen-based oral fluid samples – the authors reported the best performance from a combination of the MagMAX-96 Pathogen RNA/DNA kit and the RealPCR*M hyo DNA Mix (Poeta Silva et al., 2022).

Advances have also been made in molecular detection of the uncultivable haemoplasmas (reviewed in (Quiroz-Castañeda et al., 2020)). Giemsa staining has been used to detect *M. suis* within swine erythrocytes (Stadler et al., 2021), but the sensitivity of this test has been compared poorly against a TaqMan qPCR assay targeting the 16S rRNA gene (Guimaraes et al., 2011b; Normand et al., 2020). Given the likely underdiagnosis of swine haemoplasmas and relatively limited data available on their epidemiology, further development and field validation of haemoplasma diagnostics – particularly, new assays capable of differentiating *M. suis*, *M. parvum*, and the newly identified *C.* M. haemosuis (discussed in more detail in Epidemiology) – will be important for monitoring the spread of these diseases and identifying associated risk factors.

Serological diagnostics

Serodiagnostics are relatively simple and low-cost compared to molecular methods, making them an attractive choice for rapid herd-level diagnoses and disease surveillance studies. Several *Mhp* ELISAs are commercially available, and new serological assays continue to be reported sporadically in the literature (MaoJun et al., 2016). Poeta Silva *et al.* compared the efficacy of six commercial *Mhp* ELISAs against PCR under experimental and field conditions, reporting the highest performance from the BioChek SK108 Mhyo and IDEXX *M. hyo* Ab test ELISAs (which both detect anti-P46 antibodies) and the Hipra Civtest Suis Mhyo (a two-well indirect ELISA) (Poeta Silva et al., 2020).

Loreck *et al.* reported their development of a miniaturised protein microarray able to simultaneously detect IgG against ten swine pathogens including *Mhp*, achieving 98% diagnostic sensitivity and 80% specificity for this pathogen compared to the IDEXX ELISA (Loreck et al., 2019). These authors then applied this microarray to high-throughput screening of swine meat juice and serum samples; subsequent receiver operating characteristic analysis showed a relatively high area under the curve (AUC) value of 0.84 for *Mhp* in serum samples (Loreck et al., 2020).

DIVA is critical for monitoring *Mhp* vaccination programmes and understanding the true prevalence of naturally infected animals. However, many commonly used commercial ELISAs for *Mhp* diagnosis are unable to distinguish vaccinated pigs (Clavijo et al., 2021a; Meens et al., 2010; Scalisi et al., 2022), and the development of DIVA-compatible serodiagnostics has therefore been an active research area over the past ten years. Bai *et al.* tested the DIVA capability of a previously published indirect ELISA for secretory IgA (sIgA) against *Mhp* (Feng et al., 2010), reporting that sIgA was detected only in experimentally infected unvaccinated pigs and could be used to distinguish them from vaccinated

animals (Bai et al., 2018). Meanwhile, it was previously reported that serum from bacterin-immunised pigs did not react with the *Mhp* immunogenic lipoprotein Mhp366 (Meens et al., 2010), and this protein was later used to develop indirect ELISAs for discriminating between porcine convalescent sera and sera from bacterin-immunised pigs (Ding et al., 2021, 2019; Tian et al., 2021).

Notably, the dynamics of *Mhp* infection (characterised as slow and chronic, with a delayed antibody response by the host animal) and the variability of *Mhp* surface antigens can lead to false negatives from serological tests (Bogema et al., 2012; Gomes Neto et al., 2014; Petersen et al., 2016). A microarray study of cross-reactivity also reported that *Mfl* infections could produce false positive responses from *Mhp* serodiagnostics, illustrating the need for caution in selecting, validating and interpretating these assays (Petersen et al., 2016).

Sampling strategies and other developments

Tracheobronchial swabs have been validated for qPCR testing to assess herd-level prevalence and/or confirm a positive serological diagnosis of *Mhp* (Vangroenweghe et al., 2015a, 2018), and high agreement has been reported between PCR results obtained from these samples and from lung tissue (Burrough et al., 2018). A comparison of laryngeal swabs and deep tracheal catheters for the detection of *Mhp* in naturally infected pigs indicated that the latter sample type provided higher qPCR sensitivity at all tested timepoints (Sponheim et al., 2020). These authors later conducted a detailed investigation of PCR on pooled deep tracheal catheter samples for herd-level diagnosis of *Mhp*, concluding that pooling three or five samples at a time was a cost-effective strategy for detecting low-prevalence *Mhp* among experimentally infected pigs (Sponheim et al., 2021).

A PCR study of tonsil samples indicated that detection of *Mhr* in pre-weaning piglets could predict the development of lameness later in production (Roos et al., 2019). This group also analysed oral fluids collected from swine pens in the USA, finding that detection of *Mhs*, but not *Mhr*, in this sample type was associated with lameness (Pillman et al., 2019).

Jenvey *et al.* reported substantially higher diagnostic sensitivity in detecting anti-*Mhp* antibodies in colostrum (75%) vs. serum (45%) via the commercial Ingezim M.Hyo Compac Blocking ELISA (Jenvey et al., 2015).Finally, Jenvey *et al.* reported substantially higher diagnostic sensitivity in detecting anti-*Mhp* antibodies in colostrum (75%) vs. serum (45%) via the commercial Ingezim M.Hyo Compac Blocking ELIZA (Jenvey et al., 2015).

Efforts have been made to mitigate the substantial difficulties of culturing *Mhp*, which along with other mycoplasmas is notoriously fastidious and is often overgrown by *Mhr* in primary culture (reviewed in (Kobisch and Friis, 1996)). Cook *et al.* developed a solid medium for the culture of *Mhp* from *Mhr*-contaminated clinical samples, including purified agar and DEAE-dextran in Friis medium (Kobisch and Friis, 1996) and using a low concentration of kanamycin to selectively eliminate the less-resistant *Mhr* (Cook et al., 2016). Burgos *et al.* recently reported their development of a serum-free medium capable of supporting large-scale *Mhp* production (e.g., for vaccines), using the J strain to demonstrate proof-of-concept (Burgos et al., 2023).

Meanwhile, Pereira *et al.* compared two fluorescence-based methods – immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH) – for the detection of *Mhp* and/or *Mhs* in lung tissue, reporting higher diagnostic sensitivity (~76% vs. 40%) and specificity (100% vs. 73%) by FISH (Pereira et al., 2017). *In situ* hybridisation methods have also been combined with next-generation sequencing to facilitate associations between specific genome sequences of emerging swine pathogens (including *Mhr*) and the histopathological lesions they cause (reviewed in (Resende et al., 2019a)).

Finally, some studies have investigated potential biomarkers that could indicate *Mhp* infection in pigs. Nair *et al.* profiled serum metabolites in experimentally infected pigs, reporting a significant increase in α-aminobutyric acid and long-chain fatty acids at two and three weeks post-inoculation (Nair et al., 2019a).

Pathogenesis

In the field, pigs are commonly infected with more than one species of mycoplasma at a given time; this can make it difficult to distinguish between the pathogenic effects caused by the different organisms, or by the interactions between them. Accordingly, many studies published over the past decade have focused on *in vitro* or *ex vivo* characterisation of co-infections between two mycoplasmas (generally including *Mhp*), including the ways in which different co-infections can affect the severity of clinical signs.

In vivo *and* ex vivo *studies*

A large study of 400 lungs from pigs slaughtered in Brazil identified a significant correlation between lesion severity and the level of infection with *Mhp,* but not with *Mfl*, and a negative correlation with the level of *Mhr;* interestingly, lesion severity only positively correlated with *Mfl* and *Mhr* co-infection, and negatively correlated with *Mhp* and *Mfl* co-infection, suggestive of competition between the pathogens (Ferreira et al., 2021). Recent data from fattening pigs in the field showed that *Mhr* infection was more common in the presence of *Mhp* but confirmed that lesion severity did not correlate with co-infection by these pathogens, rather being associated with the presence of mixed-genotype *Mhr* infection (Tonni et al., 2022). This conclusion was also reached by Michiels *et al.* in their study spanning three pigs herds in Belgium (Michiels et al., 2017b). Similarly, Fourour *et al.* did not find any relationship between lesion severity and *Mhp-Mhr* co-infection, but did report worse lesion scores in specific-pathogen-free piglets experimentally infected with *Mhp* and *Mfl;* however, pigs co-infected with *Mhp* and either *Mfl or Mhr* exhibited significantly slower growth than those infected with only *Mhp*, indicative of an additive effect on their health (Fourour et al., 2019a).

Alongside pathogen co-infection or genetic diversity, emerging evidence suggests that early-life microbiota may influence *Mhp* susceptibility in the first few months of age: Nair *et al.* found that differences in the gut microbiota of piglets at three weeks of age correlated with the extent of lung lesions after experimental challenge with *Mhp* five weeks later; specifically, richness in bacteria within the short-chain-fatty-acid-producing taxa Ruminococcus_2, as well as general bacterial diversity, were significantly associated with lower lesion scores (Nair et al., 2019b), though the underlying mechanisms are not yet clear.

Mhr commonly colonises the respiratory tract of pigs yet does not always cause disease. Thus, understanding the factors determining the variable pathogenicity of *Mhr* is an active area of research: differences in virulence between strains of *Mhr* are known and have been recently experimentally evidenced (J. Wang et al., 2022a), while *Mhr* and not *Mhp* seropositivity has been implicated in the pathogenesis of pneumonia during co-infection with PRRSV (JungAh et al., 2016). *Mhr* may also spread outside of the respiratory tract and can cause systemic inflammation in the form of polyserositis and/or arthritis of the joints. Moreover, Bünger *et al.* presented a case series of eight piglets from four different farms in Austria, which had presented with central nervous system symptoms and had tested positive for *Mhr* alone at post-mortem; these data represent the first evidence that *Mhr* might be a

cause of meningitis in swine (Bünger et al., 2020). A single case report also documented *Mhr* as the sole pathogen isolated in an incident of piglet pericarditis (Ustulin et al., 2021).

Although clinical characterisation of *Mhp* infection is well established, the latest molecular detection techniques now enable direct studies of the colonisation, dissemination and persistence of the mycoplasma within the host during the course of infection. Using qPCR, Almeida *et al.* found that intra-tracheally inoculated *Mhp* strain 232 preferentially colonised the lower respiratory tract, with levels of the mycoplasma peaking at day 28 post-inoculation and persisting until the end of the experiment on day 56; moreover, levels of *Mhp* in bronchoalveolar lavage fluid at days 14 and 21 post-inoculation were significantly positively correlated with the extent of microscopic lesions on day 56 post-inoculation (Almeida et al., 2021). These data confirmed and broadened the conclusions of the group's previous work showing that the *Mhp* load correlated positively with lesion severity at all timepoints tested during experimental infection, and that the mRNA expression level of the cytokines IL-6 and IFN-γ significantly positively correlated with lung lesion score (Almeida et al., 2020).

In vitro *studies*

Potential insight into the factors underpinning the differential virulence of *Mhp, Mfl* and *Mhr* have been gleaned by the integration of genome-wide metabolic network analysis, metabolomic experiments, and secretome studies. Regarding their metabolic features, Ferrarini *et al.* highlighted key differences in glycerol metabolism that are associated with the ability of *Mycoplasma* species to produce cytotoxic H_2O_2 and could be a primary reason for the virulence of *Mhp* and *Mhr*; alongside, the authors detected differences in the metabolism of myo-inositol, amino sugar, and carbohydrates, which could explain the tissue specificity of the different pathogens (Ferrarini et al., 2016). Trueeb *et al.* also shed light on the differential virulence of *Mhp* and *Mhr* by focusing on the identification of species-specific genes that were non-essential for their growth in culture: among these genes, those involved in metabolism stood out, with *Mhp* again uniquely possessing genes involved in myo-inositol and sialic acid pathways, which could enable it to use additional/alternative energy metabolism pathways (Trueeb et al., 2019). This is one of few studies in which *Mhp* mutants have been successfully generated, due to the challenges of working with this fastidious organism: another notable success was the creation of site-specific P97-mutants of *Mhp* by Clampitt *et al.* using recombination (Clampitt et al., 2021).

Further work has added to our understanding by comparing the secretomes of a newborn pig tracheal cell line (NPTr) following exposure to pathogenic and non-pathogenic strains of *Mhp* or to *Mfl,* and the secretomes of the mycoplasmas themselves: the authors found that the secretion of known and putative virulence factors by pathogenic *Mhp* correlated with the production of damage-associated molecular patterns and extracellular proteases, consistent with cell death, in NPTr cells, while *Mfl* secreted approximately 50% fewer proteins and did not cause cell death during *in vitro* infection of these cells (Leal Zimmer et al., 2019). Interestingly, a preceding study that characterised the secretomes of *Mhp* and *Mfl* cultured in medium identified far fewer proteins, yet similar patterns emerged: *Mhp* exhibited a larger and more complex secretome, with significantly more known and putative virulence factors including adhesins, methylases, nucleases, and antigenic lipoproteins; while *Mfl* secreted just two (Paes et al., 2017a), despite the two species having a much higher level of genomic and transcriptional similarity (Siqueira et al., 2014).

Proteomic studies have also contributed to our knowledge of virulence determinants among mycoplasmas affecting swine. Yu *et al.* compared the proteomes of virulent and attenuated *Mhp* strains, identifying seven putative virulence factors with significantly differential abundance between the strains; the most highly differentially expressed was fructose-1,6-bisphosphate aldolase, which bound fibronectin and mediated attachment of *Mhp* to swine tracheal epithelial cells *in vitro* (Yanfei Yu et al., 2018a). Alongside, the effects of post-translational proteolytic processing of adhesion-related surface proteins upon pathogenicity have begun to be elucidated. Building on their previous work that characterised the proteomes of *Mhp* and *Mfl* (Paes et al., 2018), researchers working in Brazil have compared the processing of five orthologous adhesion-related proteins in pathogenic and non-pathogenic strains of *Mhp* and in *Mfl*, identifying distinct antigenic proteoforms that could be involved in the differential virulence of the strains/species (Machado et al., 2020).

One study has focused on the effects of *Mhp* infection upon the transcriptome of host NPTr cells, identifying over 1200 genes as well as 170 miRNAs whose expression was significantly affected by infection; among these, the authors highlighted increased expression of genes associated with redox homeostasis and antioxidant defence, and decreased expression of those involved in cytoskeleton and ciliary function (Mucha et al., 2020).

The means by which pathogens make their initial contact with host cells and begin to invade host tissues are critical to dissect and understand, as they can give key insights into preventative measures. Initial work identified the variable lipoprotein family as *Mhr* adhesins in porcine cell lines (Xiong et al.,

2016); more recent studies reported that the *Mhr* surface-bound glycolytic enzyme, enolase, can specifically and strongly bind porcine plasminogen and the extracellular matrix (ECM) protein fibronectin and can mediate adhesion to the PK-15 porcine kidney cell line (J. Wang et al., 2022b). The same group also characterised the *Mhr* surface proteins GAPDH (Jia Wang et al., 2021) and DnaK (which belongs to the HSP-70 family) (Y. Li et al., 2022) and shed further light on the variable lipoprotein family (J. Li et al., 2022a), finding that they too bound host plasminogen, as well as several ECM proteins; moreover, DnaK was itself recognised by sera from pigs immunised with an *Mhr* bacterin, suggesting its natural immunogenicity (Y. Li et al., 2022).

Similar *in vitro* characterisation of the *Mhp* virulence factor NADH-dependent flavin oxidoreductase revealed its binding to a porcine bronchial epithelial cell line, in which it induced oxidative stress and apoptosis pathways, as well as its interaction with plasminogen and fibronectin (Xie et al., 2021); NADH oxidase from *Mhp* behaved similarly in the same assays, and additionally induced lactate dehydrogenase release from the bronchial epithelial cells (Hao et al., 2022b). These data complemented previous work by the same group, showing that elongation factor thermo unstable (EF-Tu) acts as a novel adhesin on the *Mhp* surface *in vitro* (Yanfei Yu et al., 2018b). The ability of *Mhp* to bind fibronectin may be critical: early work showed that *Mhp* could induce fibronectin deposition on a PK-15 cell monolayer and co-localised with deposits of fibronectin on the ciliated epithelium of the airway at the site of infection *in vivo* (Raymond et al., 2015), while a follow-up study by the same group revealed that fibronectin binding allowed *Mhp* to interact with integrin β1, allowing the pathogen to gain entry into PK-15 cells and establish an intracellular niche (first within recycling endosomes, and then in the cytoplasm) (Raymond et al., 2018c). Alongside, the same group evidenced the ability of *Mhp* to bind extracellular actin on PK-15 cells, which was critical for efficient adhesion to, and infection of, these cells (Raymond et al., 2018b); while Jarocki *et al.* showed that MHJ_0125, a leucine aminopeptidase, is expressed on the surface of *Mhp* and binds heparin, foreign DNA, and plasminogen, thereby acting as a novel adhesin (Jarocki et al., 2015). Lastly, Ni *et al.* observed that *Mhp*-derived lipid-associated membrane proteins induced apoptosis in a porcine lung epithelial cell line via activation of caspases -3 and -8, and stimulated the production of nitric oxide and superoxide, which could contribute to pathogenicity (Ni et al., 2015). Similarly, *Mhp* putative type I signal peptidase induced apoptosis in PK-15 cells via caspase-3 activation (Paes et al., 2017b).

These types of proteomic analyses can also be used to compare pathogenic and non-pathogenic strains arising from the same parental mycoplasma, as a way of identifying virulence determinants. Li *et al.* found that the pathogenic *Mhp* 168 strain and its passage-attenuated equivalent, 168L,

expressed significantly different levels of 70 proteins in culture: among these were proteins involved in myo-inositol metabolism, which were more highly expressed by strain 168L, while strain 168 expressed higher levels of proteins involved in nucleoside metabolism which might help to defend against damage from host-derived reactive oxygen species (Li et al., 2019).

While these studies are important and interesting, there is a note of caution on the use of *in vitro* approaches to generate broad conclusions. This is elegantly illustrated by a study that compared the transcriptomes of *Mhp* grown in culture and isolated from the lungs of experimentally infected pigs, showing that *in vivo Mhp* upregulated the expression of 22 genes including those encoding the mycoplasma F1-like ATPase, and genes involved in nucleotide metabolism, spermidine transport and glycerol-3-phoshate transport; while simultaneously the mycoplasma population down-regulated expression of 30 genes with products related to glycerol uptake, cilium adhesion, cell division and myo-inositol metabolism (Kamminga et al., 2020). These data urge caution in extrapolating conclusions from *in vitro*-generated results to the *in vivo* setting without additional validation; this is especially important when the proposed use of the insight is towards improved strategies for vaccination or treatment.

M. suis is a less well-studied mycoplasma affecting pigs that can cause anaemia and associated productivity losses (Stadler et al., 2014), accompanied by generalised immunosuppression (do Nascimento et al., 2018), which could be particularly relevant in cases of co-infection with other pathogenic mycoplasmas. Therefore, understanding the interactions of *M. suis* with its target porcine red blood cells is a potentially important area of research. Song *et al.* built on previous work that identified a novel potential adhesion factor within the *M. suis* genome, o-sialoglycoprotein endopeptidase (Guimaraes et al., 2011a), showing that this protein could bind Band3 and glycophorin A on porcine erythrocytes, and thereby mediate adhesion of *M. suis* to its target cells (Song et al., 2018). Similarly, *M. suis* protein MSG1 (latterly re-annotated as GAPDH) was shown to bind β-actin *in vitro* and on porcine red blood cells (Zhang et al., 2015)

Finally, original genomics studies of *Mhp* shed valuable light on its biology and pathogenesis and the identity of potential virulence factors, many of which have since been confirmed by experimental study. However, since the original annotation was conducted, knowledge has moved forward; re-annotation in light of these advances is now a productive line of work. A recent study improved the level of *Mhp* genome annotation from 65% to 87%, identifying novel putative virulence factors among the 244 proteins of previously unknown function and validating their findings by comparative studies

in pathogenic and non-pathogenic strains; this study also identified putative epitopes within each of the 144 proteins of unknown function that were predicted to be surface-located (Tavares et al., 2022).

Immunology

Immunological research into mycoplasmas affecting swine has been highly productive in recent years. The field has made marked advances in our understanding of immunogenetics which have led to the breeding and detailed characterisation of resistant lines and the discovery that these lines may also respond better to vaccination, further increasing their protection from disease. Alongside, studies of the innate and adaptive responses to infection have furthered our insight into protective and pathologic mechanisms operative during infection with *Mhp* and have identified potential candidate antigens and epitopes for inclusion in novel vaccine strategies.

Of note, while some studies have cautioned about the risks of inferring between model and natural host species, or between immortalised cell lines and primary cells, other *in vitro* experiments have indicated novel mechanisms of immune evasion that could be highly relevant *in vivo* – for example, the discovery that *Mhp* may be able to both induce and then degrade macrophage and neutrophil extracellular traps as a source of nucleotides to support its own replication (Henthorn et al., 2018; Peng et al., 2019) represents an exciting step forwards in our knowledge of this pathogen.

Immunogenetics

Some breeds of pig are more susceptible than others to *Mhp* infection, and considerable advances have been made towards understanding the basis of resistance versus susceptibility, especially for the Landrace breed. Researchers working in China and Japan have reported important, stepwise progress towards not only generating a line of Landrace pigs more resistant to mycoplasmal pneumonia of swine (MPS), but also towards understanding the immunological and genetic basis of their increased resistance. After selective breeding over five generations for increased MPS-resistance, as well as high meat production, Kadowaki *et al.* observed significant decreases in MPS severity, indicating a heritable genetic basis (Kadowaki et al., 2012). Interestingly, when the researchers investigated immunological characteristics of the resistant line, they found that they had a significantly higher proportion of granulocytes among total blood leucocytes and exhibited lower recall PBMC proliferative responses *ex vivo* following vaccination against *Mhp* compared to non-resistant pigs (Shimazu et al., 2013), as

well as higher IFN-γ in blood following *Mhp* vaccination (Sakuma et al., 2020). Moreover, the group then identified that the ratio of granulocytes to lymphocytes in peripheral blood, as well as higher phagocytic activity, were key immune correlates of MPS resistance in these pigs (Okamura et al., 2016). These traits were partially linked to the LR-0.13 and LR-0.23 swine leucocyte antigen haplotypes (Ando et al., 2016). Delving deeper into the genetic basis for the difference in resistance seen between the lines, Uemoto *et al.* conducted an SNP- and haplotype-based genome-wide association study, identifying several genomic regions associated with immune parameters as potentially important: their analysis highlighted regions involved in granulocyte-to-lymphocyte ratio, cortisol levels in blood, and activity of the complement alternative pathway in serum; within these were included genes related to Th1 and Th17 responses as well as NK cell activity and the acute phase protein response (Uemoto et al., 2021). Alongside, the group identified higher levels of IL-12p40 expression in the hilar lymph nodes, lung, and spleen of the resistant line compared to controls, during experimental infection, which correlated with lower lesion scores (Sakuma et al., 2020).

Borjigin *et al.* also studied MPS-resistant Landrace lines, finding significantly higher levels of salivary IgA, TLR2 and TLR4 and of IFN-γ – as above – in their blood, and lower frequencies of B cells, after bacterin vaccination against *Mhp* (Borjigin et al., 2016a). Importantly, preliminary evidence suggests that crossbreeding of such MPS-resistant Landrace pigs with less-resistant breeds could allow transfer of favourable immunological traits (Borjigin et al., 2016c), and a partial increase in MPS resistance (Borjigin et al., 2017), to the resultant offspring.

Similar studies have also begun in other breeds of pig. Comparative transcriptional profiling of the lungs of pigs from the more susceptible Jiangquhai and less susceptible Duroc breeds revealed higher expression of *CXCL10*, *CCL4*, *IL6* and *IFNG* genes in the latter at four weeks post-infection with *Mhp*, which likely reflects a more effective immune response in the resistant breed (Ni et al., 2019). However, attempts to identify genomic regions linked with the production of higher titres of *Mhp*-specific antibodies in commercially reared groups of Landrace-cross-Large White sows did not identify any strong associations (Sanglard et al., 2020). Similarly, attempts to generate an *Mhp*-resistant Large White pig line based on PBMC immune responses proved so far unsuccessful, with the selected line exhibiting a higher level of pulmonary lesions compared to the non-selected controls (Borjigin et al., 2016b).

Alongside, other studies have enhanced our understanding of the role of immunogenetics in antibody responses to vaccination. Blanc *et al.* identified two genomic regions in which SNPs mapped to

immunity-related genes that were associated with antibody levels at three weeks post-vaccination, as well as significant differences in the blood transcriptome in low-, high- and enduring- responders, including pathways related to antiviral immunity and dendritic cells (Blanc et al., 2021). There is some evidence that the gut microbiota of piglets at the time of vaccination could additionally help predict the level of antibody response to *Mhp* vaccines (Munyaka et al., 2020).

Innate immunity

Identifying and characterising the innate immune cells present locally during infection of the lung with *Mhp* is key to understanding the initiation of immunity and of potential immunopathology. Nueangphuet *et al.* collected lung tissue from naturally infected pigs and identified IL-8-expressing neutrophils and M2-polarised IL-10-producing macrophages in the intra-alveolar space and in lung lesions, respectively (Nueangphuet et al., 2021). Interestingly, IL-8 expression was previously correlated with the development of lung lesions in pigs experimentally infected with *Mhp* (Almeida et al., 2020), while the role of IL-10 in the *Mhp*-infected lung is less clear.

Studies of porcine immune cells have also been conducted to try and understand the difference in pathogenicity of different species of mycoplasma. By exposing porcine PBMC *ex vivo* to live and inactivated *Mhp* and *Mhr* at a range of MOIs, Trueeb *et al.* identified immune signatures that were common and specific to these more- and less-pathogenic bacteria, respectively (Trueeb et al., 2020). Their study showed that an MOI >100 of live or inactivated mycoplasmas activated TNF-α production by monocytes, while *Mhp* was significantly less able to activate $CD21^{+}IgM^{+}$ B cells than was *Mhr*, and that both mycoplasmas induced CD40 upregulation by conventional and plasmacytoid dendritic cells (DCs) (Trueeb et al., 2020). These findings raise interesting questions warranting further investigation *in vivo* and/or with a focus on more physiological levels of pathogen exposure.

The interaction of mycoplasmas with DCs is especially important to understand, given these cells' pivotal role in initiating and shaping immune responses to pathogens. Fourour *et al.* exposed porcine bone marrow-derived DCs (BMDCs) to varyingly virulent strains of *Mhp*, *Mhr*, and/or *Mfl in vitro*, individually or in pairs, at an MOI approximating 50, and compared the expression of key cytokines as well as measuring BMDC viability (Fourour et al., 2019b). Interestingly, they found that all tested strains initiated the production of IL-6, IL-8, IL-10, IL-12, and TNF-α by porcine BMDCs; however, *Mhp* induced lower IL-12 production than the other species, which would be expected to favour a Th1 response, while *Mfl* exhibited highest induction of TNF-α which could initiate pulmonary inflammation

(Fourour et al., 2019b). The combination arm of the study revealed that *Mhp* and *Mfl* co-exposure led to significantly increased IL-10 production, which the authors speculated might downregulate the immune response and favour pathogen persistence *in vivo* (Fourour et al., 2019b). These data partially align with earlier work that also detected high expression of IL-10 in porcine BMDCs exposed to *Mhp in vitro,* which was accompanied by decreased expression of IL-12 and IFN-γ, together suggesting promotion of a Th2 type adaptive response (Shen et al., 2017), though these findings have yet to be translated to the *in vivo* setting.

In vitro studies in porcine cell lines have also identified potentially relevant interactions between mycoplasmas affecting swine and components of host innate immune pathways. For example, Wei *et al.* infected the 3D4/21 porcine alveolar macrophage (PAM) cell line with *Mhp* and observed activation of the nucleotide-binding oligomerization domain-containing protein (NOD)1-RIP2 signalling pathway, possibly through NOD recognition of the *Mhp* protein Mhp1; knock-down of NOD1 led to decreased activation of the TRIF-MYD88 pathway, coupled with lower transcription of the gene encoding TNF-α (Liu et al., 2022). However, given the recently documented significant genetic differences between primary PAMs and the 3D4/21 PAM cell line (X. Li et al., 2022), any data generated in this system must be interpreted with extreme caution and will require further validation in primary cells.

Adaptive immunity

Characterising the local adaptive immune response is as important as understanding innate immunity at the site of infection and provides required background knowledge for the rational design of vaccines with durable responses. Towards this aim, Li *et al.* reported that the mucosal and systemic antibody response to experimental infection of pigs with *Mhp* was dominated by IgA from ten days post-infection; they also observed co-localisation of IgA-producing cells with $CD11c^+$ DCs and macrophages in the lung and hilar lymph nodes (Henthorn et al., 2018; Peng et al., 2019). Using murine cells cultured *ex vivo*, the authors then identified a role for TLRs 2 and 4 in DC- and macrophage-mediated stimulation of *Mhp*-specific IgA-producing B cells (Li et al., 2020), which warrants follow-up study in porcine cells.

Taking a different approach to understanding the antibody response to *Mhp*, Petersen *et al.* showed that mycoplasma recombinant protein arrays can be used to gain additional insight: using pig sera from naturally or experimentally *Mhp*-infected animals, they found that the previously reported potential vaccine antigen P97 was poorly recognised, while several P97/P102 paralogues were

recognised more strongly (Petersen et al., 2019). The authors also noted key differences in antigen recognition by the porcine sera used in this study and those reported in studies of antigen recognition by sera from rabbits used in *Mhp* vaccine trials (Petersen et al., 2019); these data urge caution in extrapolating results from one species to another. Together, the characterisation of the antibody repertoire during infection is important to gain insight into immunodominant antigens, and therefore guide rational vaccine development.

More recently, Ning *et al.* screened 27 surface/secreted *Mhp* proteins for their recognition by sera from convalescent naturally infected pigs or from vaccinated animals; 24 of those proteins were recognised by at least one convalescent serum sample and 15 were recognised by at least six, among which Mhp367 and Mhp677 were bound by all convalescent samples (Ning et al., 2020). Interestingly, the recognition profile of sera from vaccinated animals was also highly variable and somewhat different, but consistently these sera did not react with Mhp462, while seven out of 11 convalescent sera did (Ning et al., 2020); this could form the basis of a differential test for animals with a history of infection versus vaccination (DIVA). In addition, during their reverse vaccinology approach, Li *et al.* identified 35 non-redundant B cell epitopes across four immunodominant *Mhp* antigens, including three adhesins and one membrane nuclease (Li et al., 2022). A similar study by Zhu *et al.* highlighted the 17-amino acid core epitope of the immunogenic *Mhr* protein P37 (Zhu et al., 2019). Together, these studies have generated a list of potential epitopes/antigens on which further study and/or the development of subunit and vectored vaccines could be based.

In the case of *Mhr,* sub-clinical colonisation of pigs is commonly observed in the field, but the immunological mechanisms underlying persistent infection are poorly understood. Merodio *et al.* established asymptomatic infection in caesarean-delivered colostrum-deprived piglets by inoculating them four times with a field isolate of *Mhr,* and detected specific IgA production at two weeks post-infection, with IgG appearing two weeks later; neither of these antibody sub-classes cleared the infection during the six weeks of study (Merodio et al., 2021). While this may be a useful model to study some aspects of asymptomatic infection, it should be borne in mind that the means of establishing this state do not resemble those of field conditions, and therefore additional validation of data generated in this system is warranted.

Maternal immunity

Lauristen *et al.* found that piglets born to sows infected with *Mhs* received significant protection from challenge at 4.5 weeks of age, which was partially attributed to passively transferred maternal antibodies; some of these piglets also mounted a PBMC proliferative response to the pathogen, but the relationship between this response and protection was not clear (Lauritsen et al., 2017). By comparison, immunologically naïve piglets exhibited high frequencies of *Mhs*-positive tissues and overt disease (Lauritsen et al., 2017). More recently, researchers have uncovered evidence of maternal *Mhp*-specific T cells being transferred to offspring via milk (Biebaut et al., 2021), though the relative roles of antibodies and cell-mediated immunity in protecting offspring has yet to be defined.

While maternally derived immunity can be useful in protecting very young offspring, the presence of immune cells or antibodies may interfere with successful vaccination of these animals. However, a recent study of the effects of maternal immunity on vaccine responses concluded that IgA^+ B cells transferred from the sow could play a positive role in response to *Mhp* vaccination at one week of age (Martelli et al., 2021). This is broadly in support of the preceding literature evidencing the lack of interference by maternally derived antibodies on vaccination of young piglets against *Mhp* (Martelli et al., 2006; Wilson et al., 2013).

Immune evasion

Few studies have focused on *Mhp* immune evasion strategies, though there is ample evidence for their existence. *Ex vivo* experiments suggest that *Mhp* is able to adhere to, but evade phagocytic uptake by, primary PAMs (Deeney et al., 2019). However, a different study using primary PAMs alongside the 3D4/21 PAM cell line showed that intracellular *Mhp* significantly inhibits cellular autophagy during infection by inducing autophagosome formation and preventing lysosome fusion, thereby promoting its own survival and proliferation (Wen et al., 2022). In porcine PBMC, exposure to *Mhp*-derived lipid-associated membrane proteins led to high levels of nitric oxide and ROS in the supernatant, and attendant increases in monocyte and lymphocyte apoptosis (Bai et al., 2015). Thus, *Mhp* may exert distinct immune-evasive actions on PAMs and monocytes/lymphocytes in the blood.

Interesting data have also emerged about a possible interaction of *Mhp* with macrophages and neutrophils. An *in vitro* study using a human cell line showed *Mhp'*s potential ability to degrade the extracellular chromatin used by macrophages and neutrophils to form extracellular traps (NETs), using

the traps as a source of nucleotides for DNA replication (Henthorn et al., 2018). Later work with primary porcine neutrophils showed that *Mhp* exposure induced the formation of NETs *in vitro* which were rapidly degraded, at least partly due to the action of the Mhp597 nuclease (Peng et al., 2019). These data point to a potentially important mechanism of immune evasion that directly supports mycoplasma growth, and so warrants further investigation *in vivo*.

Lastly, although *Mhp* is well-known to induce ciliary paralysis in the respiratory tract, thereby reducing pathogen clearance, few studies have shed light on this phenomenon. A notable exception is the work of Jaroki *et al.* who showed that two surface-exposed peptidases can degrade several peptides that together regulate ciliary beat frequency, inflammation and innate immunity in the lung: bradykinin, substance P, neurokinin and neuropeptide Y (Jarocki et al., 2019a). These peptidases could therefore represent key colonisation- and persistence- promoting factors of *Mhp.*

Geographic distribution and epidemiology

Given the clinical significance of *Mhp* infections, this pathogen has unsurprisingly dominated the field of swine mycoplasma epidemiology research over the past decade. This section will therefore deal primarily with *Mhp*, while also including a handful of studies that have addressed the epidemiology of *Mhr* and *Mhs.* Numerous studies have also begun to address knowledge gaps in the epidemiology of the haemoplasmas (particularly *M. suis*, the causative agent of infectious anaemia in pigs). Previously believed to be rickettsial organisms, 16S rRNA gene sequencing of haemoplasmas has led to their reclassification as mycoplasmas (reviewed in (Tasker, 2022)), and our understanding of the prevalence and epidemiology of haemoplasma infections has slowly increased over the past decade.

Global situation

The Americas

Mhp is widespread in North and South America, and its presence has been significantly associated with swine mortality in wean-to-finish systems in the USA (Magalhães et al., 2022). A report on diagnostic cases submitted to the Iowa State University's Veterinary Diagnostic Laboratory presented ~27% PCR-positivity for *Mhp*, with higher prevalence observed in the autumn season and in finishing-age pigs (Rawal et al., 2018). Meanwhile, *Mhs* was the most common cause of swine joint- or leg-

associated lameness in a recent retrospective study in Iowa state (Canning et al., 2019), and the severity of this lameness was reported to vary between clinical isolates (Gomes-Neto et al., 2016).

Swine enzootic pneumonia is a similarly serious problem and frequent cause of death in pig farms of Brazil (Gonzaga et al., 2020; Piva et al., 2020) and Argentina (Cappuccio et al., 2018), particularly in combination with sIAV infection (De Conti et al., 2021; Rech et al., 2018). Biondo *et al.* studied the lungs of captive wild boar in a slaughterhouse in Brazil – signs of pneumonia were extremely common, and *Mhp* was detected in ~59% of samples (often in co-infections with *Mhs* and/or other swine lung pathogens) (Biondo et al., 2021). For domestic pigs, a recent survey of various production systems in the Central-West, Southeast, and South regions of Brazil reported ~39% prevalence of *Mhp* and a high rate of co-infections with other bacterial and viral respiratory pathogens (Balestrin et al., 2022), though much higher prevalence has been reported in slaughter pigs of South Brazil (Pacce et al., 2019).

Swine haemoplasma infections are also apparently widespread in Brazil. ~76-80% PCR-positivity was recently reported among swine in the country's South and Northeast regions (Gatto et al., 2019; Toledo et al., 2016), and up to 82% positivity was observed specifically for *M. suis* in extensive pig production systems in the state of Maranhão (dos Santos Martins et al., 2019). Petri *et al.* conducted a molecular survey of *M. suis* prevalence in finishing pigs in Minas Gerais state, finding ~60% positivity and a weak but significant correlation between pathogen burden and lower average daily weight gain (Petri et al., 2020).

Europe

As in the Americas, many recent studies from Europe have focused on assessing the prevalence of *Mhp* infections in various countries and farming conditions. *Mhp* was reported to be the main pathogen associated with pneumonia and pericarditis in Ireland (Rodrigues da Costa et al., 2020) and with mycoplasma-like lung lesions in Spain and Portugal ((Pallarés et al., 2021); reviewed in (Maes et al., 2023)). It was the second most common cause of pneumonia in a study of Austrian pigs (after *Pneumocystis* species, with which it often co-infects) (Kureljušić et al., 2016), and Deffner *et al.* found that 75% of pen-based oral fluid samples from 16 German and Austrian pig farms were positive for *Mhp* DNA (Deffner et al., 2022). *Mhr* was also commonly detected (~79%) in the upper respiratory tracts of German domestic pigs (Bunke et al., 2020). In Belgium and the Netherlands, a study of peri- and post-weaned piglets found that *Mhp* prevalence increased with age (~7% at 3-5 weeks and ~11% at 6-11 weeks) and was negatively associated with some climatic variables (e.g., precipitation) (Vangroenweghe et al., 2015b).

In Italy, meanwhile, Giacomini *et al.* studied the dynamics of *Mhp* seroconversion in one-, two- and three-site production systems, reporting higher prevalence in older animals and in one- and two-site vs. three-site systems (Giacomini et al., 2016). *Mhr* (alone or in co-infection with *Haemophilus parasuis*) has also been reported to be a primary cause of polyserositis and the most common bacterium detected among pigs with recurrent arthritis in a high-density breeding area of Italy (Salogni et al., 2022, 2020). *Mhs* also causes severe lameness in some Italian pig farms, and frequent relapse was observed following antibiotic treatment for this condition (Moronato et al., 2017).

A study of gestating sows in ten French herds reported an average of 53% PCR-positivity for *M. suis* among these animals (Brissonnier et al., 2020), higher than the 30% prevalence previously reported in Germany (Stadler et al., 2019). In 2020, the novel haemoplasma species dubbed "*Candidatus* Mycoplasma haemosuis" (*C.* M. haemosuis) was detected in a German pig with clinical signs more suggestive of infection with *M. suis*, indicating possible pathogenic properties by this new haemoplasma (Stadler et al., 2020). This group later developed a new qPCR assay for *C.* M. haemosuis detection on a larger scale, reporting a farm-level prevalence of ~14% on piglet-producing farms and 45% on fattening farms in Germany (Ade et al., 2022).

Africa

Despite the rapid recent growth of Africa's pig production systems and the prevalence of respiratory infectious diseases within them, relatively few studies have been conducted on the epidemiology of the pathogens responsible for these diseases (reviewed in (Oba et al., 2020)). Despite containing one of the largest pig populations in Africa, Nigeria has seen few studies of enzootic pneumonia, for instance. The data that are available indicate that prevalence is high – a retrospective study of post-mortem records in Ibadan state reported ~50% incidence of pneumonia, most of which was attributable to *Mhp* infection (Olaniyi et al., 2020). Several studies have also explored the prevalence of infectious anaemia in Nigerian swine, though many of these use the old classification of *Eperythrozoon* for the causative organism (Igbokwe and Maduka, 2018; Ogbaje et al., 2015; Olaosebikan et al., 2018).

Finally, *Mhp* was the third most prevalent swine respiratory pathogen after *Metastrongylus* species helminth infections and *A. pleuropneumoniae* in a study of slaughtered pigs in Uganda (Oba et al., 2021), and a more recent study reported a significant association between the presence of *Mhp* and *Strongyles* species in this country (Oba et al., 2023).

Asia, the Middle East and Oceania

In China, the prevalence of *Mhp* appears to be increasing, with a recent analysis of clinical samples indicating a rise in positivity rate from 7.2% in 2018 to 43.8% in 2020 (H. Zhang et al., 2021). Detection rates were even higher in a study of slaughterhouses in the Shanxi province, where 77% of PCR-tested lung samples were positive for *Mhp* (Yue et al., 2021). Similarly, the prevalence of *M. suis* is also trending upwards; seroprevalence in domestic pigs of eastern China was reported as ~26% in 2014 and 38% in 2016, with cases particularly concentrated in the Shanghai region during the summer and autumn seasons (ZhongYang et al., 2017). Meanwhile, *C.* M. haemosuis was first identified in 2017 in the Zhejiang province of China, where 26.5% of clinically healthy pigs were PCR-positive for it (Fu et al., 2017).

Acharya *et al.* reported an overall *Mhp* seroprevalence of ~23% in the western districts of neighbouring Nepal, where several factors (e.g., the use of improved vs. local breeds and continuous production vs. all-in all-out systems) were associated with high risk of detection (Acharya et al., 2019). Interestingly, a seroprevalence survey in Brazil reported that all-in all-out management was associated with a decreased likelihood of detecting *Mhp* antibodies, indicating that region-specific differences (e.g., in climate, other farm management variables, etc.) may impact this particular risk factor (Baraldi et al., 2019).

In South Korea, *Mhp* was the second most common respiratory pathogen of pigs (after *H. parasuis*) in a survey of oral fluid samples from commercial pig farms, with ~68% and 88% animal- and farm-level prevalence, respectively (Cheong et al., 2017). Slightly lower farm-level prevalence (~64%) was reported in a later study utilising nested PCR on laryngeal swabs for diagnosis, where the authors identified several risk factors (e.g., larger herd size and higher gilt replacement rates) for higher *Mhp* detection rates among weaning piglets (Oh et al., 2020). *C.* M. haemosuis was also detected in a single pig in South Korea following this haemoplasma's first reported detection in China (MinGoo et al., 2019).

Limited data are available on the epidemiology of swine enzootic pneumonia in Southeast Asia, where pig farming is predominantly based in smallholder operations that may have limited access to animal health information and disease control resources. Lee *et al.* conducted a rare seroprevalence survey in Vietnam, finding 46% prevalence of *Mhp* and high levels of co-infections in swine farms of the Bac Giang and Nghe An provinces (Lee et al., 2020). *Mhp* has also been reported as a common co-infection

with porcine circovirus type 2 (PCV2) in this country (Dinh et al., 2021). Meanwhile, Thongmeesee *et al.* reported ~37% prevalence of haemoplasma infections within commercial pig farms of Thailand and identified a putative novel species via genomic analysis (Thongmeesee et al., 2022).

Surveillance, transmission and risk factors

While direct contact is the primary means of *Mhp* transmission (reviewed in (Sibila et al., 2009)), potential routes of indirect transmission remain poorly understood (Garza-Moreno et al., 2022); despite the general fragility of mycoplasmas, for instance, *Mhp* was found able to survive on dry surfaces for 8+ days in cold temperatures (Browne et al., 2017) and is known to form biofilms on abiotic surfaces in an extracellular DNA-dependent process (Raymond et al., 2018a), though the implications of *Mhp* environmental survival on its transmission remain unclear. Dam-to-piglet transmission is also thought to play an important role in the maintenance of enzootic pneumonia on commercial farms (Biebaut et al., 2022), though it has not been detected in all studies (Takeuti et al., 2017a). Insufficiently long quarantine of gilts before introduction to the herd has been hypothesised as an important risk factor for *Mhp* infection (Biebaut et al., 2022). A study of gilts in Brazil reported that most were PCR-positive for *Mhp* for 1-3 months in natural infections (though some were positive at later timepoints), with smaller *Mhp*-negative subpopulations existing within positive herds (Takeuti et al., 2017b).

As these and other studies have begun to clarify the transmission routes and patterns of *Mhp*, others have addressed certain risk factors associated with increased odds of disease occurrence and maintenance in commercial pig populations. Increasing concentrations of particulate matter were significantly associated with higher odds of pneumonia lesions, higher severity of these lesions, and greater likelihood of PCR-positivity for *Mhp*, indicating the importance of environmental respiratory factors beyond the causative bacterium itself (Michiels et al., 2015). Computational modelling has also been applied to *Mhp* epidemiology – Nathues *et al.* developed a stochastic compartmental model to simulate within-herd transmission of *Mhp* within closed pig herds and estimate the effect of various farm management and control strategies on disease severity (Nathues et al., 2016).

Meanwhile, the swine microbiome is increasingly recognised as an important factor in the progression of various diseases (reviewed in (Aluthge et al., 2019)), and this aspect of swine health has recently been addressed with regards to *Mhp* infection. In a recent study of the associations between environmental bacteria and the swine microbiome, Valeris-Chacin *et al.* reported that the presence of

Mhp was associated with reduced alpha diversity and increased levels of other pathogens and *Mhr* in swine tracheal fluids (Valeris-Chacin et al., 2021). Similar results were observed in a later analysis of commercial swine herds in Brazil, where *Mhp* infection was associated with significant changes in the composition of the lung microbiota (Sonalio et al., 2022). Experimental infection with *Mhr* has also been found to alter the components and metabolism of the swine gut microbiome (Zhang et al., 2023), and both *Mhr* and *Mhp* were significantly associated with the presence of lung lesions in a recent metagenomic sequencing study of swine lung lavage fluid samples (Li et al., 2023).

Meanwhile, surveillance for *Mhp* is often conducted via serodiagnostics on pen-based oral fluid samples, as these methods are relatively simple, cost-effective, and non-invasive to the tested animals (Clavijo et al., 2021a). However, the sensitivity of this sampling-testing combination can be too low to detect relatively small-scale transmission events (e.g., from the introduction of one *Mhp*-infected gilt to a naïve population), necessitating more invasive and labour-intensive methods to achieve adequate detection rates (Betlach et al., 2020). Inconsistent results have also been reported from PCR testing of oral fluids for *Mhp* in different pens or at different timepoints (Hernandez-Garcia et al., 2017).

In a recent field comparison of different surveillance methods, Clavijo *et al.* reported that PCR analysis of tracheal samples allowed the quickest high-probability detection of *Mhp* (Clavijo et al., 2021a). Garza-Moreno *et al.* also validated the detection of *Mhp* DNA via environmental sampling in pig farms of the Midwest USA – they reported that *Mhp* could be detected in air, swine surface swabs, and stall swabs on farms with clinical infections, while the latter two sample types were also suitable on farms with only subclinical infections (Garza-Moreno et al., 2022).

Monitoring of herd-level mortality data and antimicrobial and vaccine usage levels has been validated for surveillance on Danish swine farms, where Lopes Antunes *et al.* reported that changes in these information streams could be used as an early-warning system for the presence of *Mhp* and other respiratory pathogens prior to confirmatory diagnostic testing (Lopes Antunes et al., 2019). Alongside, in an observational study of finishing pigs, Pessoa *et al.* found evidence to support the use of coughing frequency to complement post-mortem data (e.g., the prevalence of lung lesions at slaughter) and improve on-farm respiratory disease management (Pessoa et al., 2021).

While a less economically significant pathogen than *Mhp*, *Mhr* is known to cause polyserositis and arthritis (Clavijo et al., 2017) and has also been associated with outbreaks of conjunctivitis on swine farms in the USA (Resende et al., 2019b); however, its epidemiology remains poorly understood. The

prevalence of *Mhr* on commercial pig farms has been reported to vary significantly with animal age (e.g., 8% in preweaning piglets vs. ~98% in postweaning pigs) (Clavijo et al., 2019a, 2017). Luehrs *et al.* analysed 1,375 pig lungs from Swiss and German farms with a history of chronic respiratory disease and found that, while *Mhr* was significantly more common in pneumonic lungs, its presence alone was not associated with clinical signs or lung lesions, and co-infection with *Mhr* did not appear to worsen the lesions caused by *Mhp* infection (Luehrs et al., 2017).

Several recent studies have also addressed epidemiological questions surrounding swine haemoplasmas. *M. suis* is spread primarily via parenteral exposure or bites, and the detection of PCR-positive pre-suckling piglets on German farms indicates that vertical transmission may occur as well (Stadler et al., 2019). *M. suis* has also been detected in blood-free fluids (e.g., saliva and urine) from infected animals (Dietz et al., 2014), though a later study of oral transmission indicated that the epidemiological relevance of blood-free transmission routes is minimal (Ade et al., 2021). In their analysis of *C.* M. haemosuis prevalence among German pig farms, these authors also identified PCR-positive piglets immediately after birth (prior to colostrum intake), suggesting that this newly identified haemoplasma is capable of vertical transmission (Ade et al., 2022).

Schwartz *et al.* isolated haemoplasmas including *M. suis* from stable flies (*Stomoxys calcitrans*) in Austrian pig farms, indicating that it might facilitate transmission of this pathogen (Schwarz et al., 2020). *M. suis* DNA was also detected in ~15% of sampled hog lice (*Haematopinus suis*) in Argentina (Acosta et al., 2019), and haemoplasma DNA was present in ~9% of wild boar-associated *Amblyomma* species ticks in Brazil (de Souza Santana et al., 2022). Further studies will be needed to clarify the potential roles of these arthropods in swine haemoplasma transmission.

Wildlife

Mhp is harboured by many wild boar (*Sus scrofa*) populations, where it causes similar respiratory pathology as in domestic pigs (Risco et al., 2015). The involvement of wild boars in the epidemiology of *Mhp* among domestic pigs is still poorly understood (reviewed in (Maes et al., 2018)). Studies in several countries have reported similar levels of *Mhp* seroprevalence among wild boar populations – e.g., approximately 20% in the USA (Baroch et al., 2015), 25% in Sweden (Malmsten et al., 2018), and 21% in the Tuscany region of Italy (Bertelloni et al., 2020). Interestingly, no *Mhp* antibodies were reported in a serosurvey of Greek wild boars, where the most common respiratory pathogen by far was *A. pleuropneumoniae* (Touloudi et al., 2015).

Estimates of *Mhp* seroprevalence in wild boars of Brazil have varied – Severo *et al.* reported ~20% seroprevalence (Severo et al., 2021), but a more recent survey of respiratory pathogens in Brazilian wild boars found little evidence of *Mhp* (da Silva Andrade et al., 2022). Further analyses will be needed to clarify any potential role for these animals in *Mhp* epidemiology in this region. Findings on haemoplasmas, which also infect wild boars, have been more concordant: Dias *et al.* identified 50% PCR-positivity for *M. suis* in Brazilian wild boars (Dias et al., 2019), while a later study identified similar prevalence (~59%) in both wild boars and hunting dogs in two regions of Brazil (though there was no indication of cross-species transmission) (Fernandes et al., 2022).

In Switzerland, Batista Linhares *et al.* observed ~26% *Mhp* PCR-positivity among wild boars and identified their population density and the occurrence of enzootic pneumonia in domestic pigs as significant risk factors (Batista Linhares et al., 2015). Interestingly, while these authors had hypothesised that spill-over from wild boars to domestic pigs might be responsible for ongoing sporadic outbreaks on Swiss pig farms, their data suggested that the reverse (transmission from domestic pigs to wild boars) was in fact more likely to occur (Batista Linhares et al., 2015). In studying a population of European wild boars, meanwhile, Goedbloed *et al.* reported that *Mhp* seroprevalence was higher among animals genetically determined to be wild boar-domestic pig hybrids, suggesting that the immune genes of domestic swine may increase their vulnerability to this pathogen (Goedbloed et al., 2015).

Finally, Pearson *et al.* modelled the risk of pathogen transmission from wild boars to domestic pigs in Australia, estimating that *Mhp* had the highest probability of exposure to free-ranging domestic pigs (Pearson et al., 2016).

Control of the disease

Although specific elimination programmes have been introduced, *Mhp*-specific biosecurity and control measures have not been developed, but if general strategies are followed, *Mhp*-free status and the prevention of the introduction of new strains into herds can be achieved (Maes et al., 2018). Disease control strategies for *Mhp* infections in pig herds include management and biosecurity practices, administration of antimicrobial drugs, and application of bacterins. The implementation of strict all-in-all-out production practices allows for adequate cleaning and disinfection to reduce the

bacterial load in the environment before new animals enter the premises, and thus reduces the risk of *Mhp* and other pathogen infections. Other management practices such as proper purchase policy and gilt acclimation strategies, age-segregation, optimal stocking densities, prevention of other respiratory diseases, and optimal housing and climatic conditions will contribute to the control of infection (Pieters and Maes, 2019). It has been found, however, that implementation of the above-mentioned practices, alone or in combination, generally provide only partial protection (Robbins et al., 2019). One reason for the apparent failure of the all-in-all-out management strategy to control disease was addressed by Fitzgerald *et al.*, who noted that implementation of strict all-in-all-out practices is difficult in farrow-to-finish farms as most lack facilities exclusively dedicated to slow-growing and/or sick pigs (Fitzgerald et al., 2020). This means that pigs are often regrouped at various times according to their body weight in an effort to achieve uniformity in slaughter weight. These authors proposed that an extension to the vaccination programme to include sows and gilts may be beneficial to controlling the infection spread within the herd (Fitzgerald et al., 2020).

Policy

A standardised system for classifying the *Mhp* status of swine breeding herds in the USA was developed by industry bodies with leading US 'actors', based on epidemiological and ecological features of *Mhp* at the herd level (Clavijo et al., 2021b). The authors envisage that the classification could be used as a guide for *Mhp* management by both swine producers and veterinarians to characterise the health status of farms and set realistic goals for control or elimination of the pathogen (Clavijo et al., 2021b).

A national *Mhp* elimination programme was implemented in Norway in 1994 followed by population-wide surveillance and documentation (Gulliksen et al., 2021). The strategy involved partial depopulation of all *Mhp*-positive farrow-to-feed and farrow-to-finish herds, with total depopulation in all positive finisher herds. All units and pens of affected herds were cleaned and disinfected using sodium hypochlorite or potassium peroxymonosulfate (Virkon S®). Between 1994 and 2009, 4% of individual samples and 12.4% of the herds were defined as positive, and in 2009, the Norwegian pig population was declared free from *Mhp* (Gulliksen et al., 2021). There has been ongoing surveillance, and between 2009 and 2019, a total of 44,228 individual serum samples have been analysed and found negative for the presence of antibodies against *Mhp*. This strategy's success is thought to be due to factors such as moderate-to-low prevalence of the pathogen, the well-documented and effective eradication protocol adopted, accurate diagnostic tests, and farming practices such as small herd sizes

and low herd density in most parts of the country, combined with the negligible import of live pigs. Seropositive samples detected in 2016 and 2017 were from herds vaccinating against *Mhp* (Gulliksen et al., 2021).

Although outbreaks of enzootic pneumonia in pigs caused by *Mhp* dropped drastically from >200 in 2003 to two cases in 2013 following an elimination programme implemented in 2004 in Switzerland, it was not possible to eliminate the pathogen from Swiss pig production (Overesch and Kuhnert, 2017). The *Mhp* strains involved in the outbreaks were genotyped using extended MLST to try to understand the sources and routes of infection (Overesch and Kuhnert, 2017). This typing revealed that most of the cases in 2014/2015 were due to two *Mhp* sequence types, identical to those harboured by wild boar. As there was insufficient evidence to assign wild boar as a reservoir, the authors concluded that wild boar are more likely to be the recipient rather than the transmitter of *Mhp* (Overesch and Kuhnert, 2017). They suggested that, as *Mhp* can be transmitted by air over distances of 5-10 km, implementing a monitoring scheme in wild boar in combination with genotyping of all available samples from domestic pigs and comparing the sequences against the PubMLST database of sequence types for *Mhp* could be useful in the tracing of strains on an international level to direct actions for combating enzootic pneumonia (Overesch and Kuhnert, 2017).

The elimination programme implemented in 2004 in Switzerland was based on total depopulation strategies of affected fattening farms as well as partial depopulation in breeding farms (Scalisi et al., 2022). The seroprevalence of *Mhp* in domestic pigs on 968 farms was tested 15 years later and found to be 0.98% (Scalisi et al., 2022). Several samples gave suspect results, and the authors speculated that samples that reacted weakly could be a result of cross-reactivity with antibodies mounted against *Mfl* a commensal of the porcine respiratory tract (Scalisi et al., 2022). Only one of the 968 farms enrolled in the study had a large proportion of strongly positive samples indicating an ongoing infection with *Mhp*. Seropositivity derived from vaccination could be ruled out due to Swiss legislation against vaccination and importation of vaccinated pigs. The role of wild boar as a reservoir was once again raised as the authors noted that the geographical distribution of the farms that tested seropositive for sows in their study corresponded approximately to the general distribution of reported cases in the past and overlaps in part with the wild boar population (Scalisi et al., 2022).

The costs of elimination were largely paid by farmers in the national programme in Norway, with about 40% of the analysis costs covered by the farmers, and 40–50% paid by the Norwegian Pig Health Service through collective funds from a pork levy all farmers are required to pay, with small

contributions from Norsvin SA and the Norwegian Veterinary Institute, and the final 10-20% paid by the abattoirs (Gulliksen et al., 2021). It was estimated in 2009 that the annual economic benefit of freedom from *Mhp* in the Norwegian pig population (1.5 million pigs slaughtered per year) was 7-18 million Norwegian kroner (approximately 0.65-1.68 million USD, 0.52-1.35 million GBP in 2023) (Gulliksen et al., 2021).

Control

The introduction of *Mhp* via fomites is not the highest risk for farms; the highest risk is incoming pigs (Maes et al., 2018). To improve the effectiveness of biosecurity strategies for *Mhp* disease prevention, it is important to identify the means by which the pathogen can be introduced and transmitted. A Bayesian Belief Network was employed to identify and quantify the probability of disease occurrence in Canadian swine farms (Cox et al., 2016). Pathogen introduction and transmission were increased when spilt feed (feed that did not fall directly in a feeding trough) was not cleaned up and fed to the pigs or disposed of immediately; by spreading manure from external sources onto land adjacent to the pigs; and in farms that were within 10 km (~6.21 miles) of another swine farm (Cox et al., 2016).

Gilt acclimation focused on reducing the bacterial shedding at first farrowing is used in efforts to control the vertical transmission of *Mhp*. Although the main strategy used to acclimate gilts in Europe and North America is vaccination, intentional exposure is also used as a means to limit *Mhp* infection at weaning. A survey of gilt acclimation strategies used in 18 European countries was carried out (Garza-Moreno et al., 2017). Of the 321 farms for which information was obtained, 278 had gilt isolation sites available for acclimation, and of those, 225 had specific acclimation procedures for *Mhp* (Garza-Moreno et al., 2017). Vaccination against *Mhp* was the most common acclimation procedure, followed by vaccination and animal exposure, with either culled sows or pigs, and a combination of vaccination and both types of live animal exposure (Garza-Moreno et al 2017). Although inadequate acclimation would leave gilts as a potential source of infection of offspring, only 23.7% of farms verified the acclimation process by ELISA, PCR, or both (Garza-Moreno et al., 2017).

The success of natural exposure to *Mhp* is important in gilt acclimation to prevent outbreaks and recirculation of pathogen. Intentional infection of pigs with *Mhp* in experimental settings is usually done via intratracheal inoculation, as this allows the application of infectious doses of inoculum to the pig's lower respiratory tract, promoting reliable *Mhp* colonization (Figueras Gourgues et al., 2020). However, intratracheal application is labour-intensive, time-consuming, and invasive, making

application in agricultural settings undesirable. A procedure for the intratracheal administration of lung tissue homogenate containing *Mhp* for acclimation of replacement gilts was described (Robbins et al., 2019). The procedure was tested under field conditions for the development of a herd-specific lung homogenate that encompasses herd-specific pathogen exposure (Robbins et al., 2019).

An experiment to evaluate the optimum seeder-to-naïve gilt ratio to achieve successful natural exposure was also carried out (Roos et al., 2016). *Mhp* strain 232 was used in the study as its infection dynamics are well understood, and the exposure was set by dividing the gilts into six groups of 10 with different ratios of seeder-to-naïve animals (Roos et al., 2016). The ideal ratio of six seeders per group of 10 gilts was required for successful exposure to *Mhp* in a 4-week exposure period (Roos et al., 2016). As an alternative to natural exposure as an acclimation strategy, nebulisation technology that would mimic the natural conditions of *Mhp* infection was compared to intratracheal inoculation (Figueras Gourgues et al., 2020). No statistically significant differences in the proportion of *Mhp* positives or in tracheobronchial swab pooled samples were detected between batches early after exposure via intratracheal or nebuliser inoculation (Figueras Gourgues et al., 2020). Unfortunately, the *Mhp* status of the gilts at first farrowing was not tested, and the authors were not able to determine the efficacy of the acclimation protocol in reducing bacterial shedding (Figueras Gourgues et al., 2020). Furthermore, while nebulisation may be a more convenient method to infect gilts for acclimation, it may pose some risks in terms of biosecurity and biocontainment or may be precluded by law for these reasons; therefore, further investigation would be required before introduction as a controlled exposure acclimation method.

A whole-herd partial budget model using field data to determine the economic value of programmes to eliminate *Mhp* from breeding herds in the USA was developed (Silva et al., 2019). The authors used retrospective data from 2004-2017 from 70 breeding herds that implemented herd closure or whole-herd medication protocols to eliminate *Mhp,* to determine the payback period of each. Although partial depopulation has been successfully applied in Mhp elimination programmes in Switzerland and Norway, the most common protocols in the USA are herd closure with herd medication, and whole-herd medication without herd closure. The herd closure with medication is an adaptation of the Swiss protocol that allows non-interrupting farrowing to minimise production losses (Silva et al., 2019). The whole-herd medication with no closure uses antibiotics only. Based on the estimated probabilities of success for the herd closure with medication protocol of 83%, and the medication protocol of 58%, the payback periods for eliminating *Mhp* from breed-to-finish operations was 2.18 months for the herd closure and 6.90 months for the medication protocol (Silva et al., 2019). If the authors assumed

a 100% success rate to eliminate *Mhp* using the herd closure or medication protocols, the payback periods would be 1.8 and 3.2 months, respectively. The lower success rate and higher project cost per sow (due to the cost of antibiotics) compared to herd closure made the medication protocol less feasible and the herd closure protocol economically better (Silva et al., 2019). Interestingly, a survival analysis by Yeske *et al.* compared the time-to-detection of *Mhp* in breed-to-wean farms after the application of herd closure and medication or whole-herd medication and found that farms that employed the herd closure and medication eradication method remained *Mhp*-negative for a longer period compared to whole-herd medication. The authors noted that, as this was an observational study, they were unable to separate the true efficacy of the eradication method from any potential reintroduction of the pathogen due to biosecurity breaches (Yeske et al., 2020).

The financial cost of *Mhp* positivity was estimated in 56 farrow-to-finish pig farms in Ireland using the Teagasc Pig Production Model (Calderón Díaz et al., 2020). Financial risk analysis was conducted by Monte Carlo simulation within this model using the Microsoft Excel add-in @Risk (Calderón Díaz et al., 2020). Using these methods, the authors estimated that annual mean profit was reduced by 41% in *Mhp*-positive farms. The authors note, however, that *Mhp*-positive farms were more likely to also be positive for PRRSV and/or sIAV compared to *Mhp*-negative farms, and these co-infections may contribute to the higher financial losses observed (Calderón Díaz et al., 2020). Their modelling provides evidence to support the adoption of effective disease control measures and/or to implement eradication programmes (Calderón Díaz et al., 2020). Finally, Paz-Sánchez *et al.* recommended that pigs that do not reach the optimal weight at slaughter age be sent to slaughter regardless to minimise economic losses and the risk of reinfection (Paz-Sánchez et al., 2021).

Therapeutics

The practice of metaphylaxis or strategic use of antibiotics in the absence of clinical disease or a positive *Mhp* test has been demonstrated to be beneficial in terms of pig growth and productivity, but not always in the control of pathogens (Maes et al., 2020). A study using strategic medication with Aivlosin at 2.5 mg tylvalosin/kg in drinking water (for seven days at weaning and when moved to finisher barn) resulted in increased average daily weight gain and decreased the average number of days in finishing (Pallarés et al., 2015). This treatment also significantly reduced mycoplasma-like lung lesions, and *Mhp* was not detected by qPCR in samples from lungs with lesions, in contrast to the controls that were positive for *Mhp* (Pallarés et al., 2015). Similar findings of increased average daily gains in pigs given antibiotics in feed were made in a study by Puls *et al.*, but there was no effect of

the antibiotic feeding programme on the incidence of morbidity and mortality (Puls et al., 2019). A study that involved three experimental groups receiving metaphylactic treatment with tilmicosin, valnemulin, tulathromycin, and a control group found no differences in the median of the degree of lung injury between groups; however, individuals treated with valnemulin had fewer lungs with lesions suggestive of swine enzootic pneumonia, indicating a lower incidence of co-infections (Stingelin et al., 2022). The antibiotic treatments produced better performance results, lower mortality, but there was no difference in *Mhp* DNA (copies/µL) in bronchoalveolar lavage fluid at slaughter (Stingelin et al., 2022). Although growth parameters are early indicators of subclinical or preclinical disease, strategic use of antibiotics alone may not be enough to control *Mhp*-associated pneumonia, and alternatives that do not risk the development of antimicrobial resistance may be more beneficial. Colostrum supplementation increased pig homogeneity of weight and average daily gain and decreased the use of antibiotics and mortality due to diarrhoea (Miguel et al., 2021). A study of dietary fibre intake and growth performance of piglets found that dietary fibre treatment improved the growth performance and reduced the mortality rate of piglets during the weaning period (BoShuai et al., 2018). Dietary supplementation may be preferable to metaphylaxis and its accompanying risks for resistance development in improving productivity and pig health.

Antimicrobial sensitivities of porcine mycoplasmas from different geographic regions have been tested and reported, but there are currently no clinical breakpoints available to facilitate data interpretation and correlation of MICs with *in vivo* efficacy (de Jong et al., 2021a; Maes et al., 2020). The antimicrobial susceptibilities of *Mhp* isolates associated with respiratory disease between 2010 to 2012 in Belgium, Spain and the UK were tested at a central laboratory (Klein et al., 2017). The highest *Mhp* MIC_{90} values were for enrofloxacin, marbofloxacin and florfenicol in strains from the UK. Later MIC testing of isolates collected between 2015-2016 from Belgium, France, Germany, Great Britain, Hungary, Italy and Spain found similar $MIC_{50/90}$ results (Klein et al., 2017) except for the MIC_{50} of oxytetracycline, which was more than two dilution steps higher (de Jong et al., 2021a).

Some studies have tested the antimicrobial sensitivities of *Mhp* and *Mhr*. Field isolates of *Mhp* and *Mhr* obtained from enzootic pneumonia-like lung lesions during 2009-2011 in Korea were compared to reference strains *Mhp* (ATCC 25934) and *Mhr* (ATCC 27717) (JiSung et al., 2016). Korean *Mhp* and *Mhr* isolates are highly susceptible to tylvalosin but somewhat resistant to chlortetracycline (JiSung et al., 2016). Hungarian *Mhr* strains isolated between 2014 and 2017 were most sensitive to tetracyclines and pleuromutilins (Bekő et al., 2019a). Field isolates of *Mhp*, *Mhr* and *Mhs* from clinical samples collected in Southern European countries between 2013 and 2018 were most sensitive to tylvalosin

and valnemulin but resistant to lincomycin (Rosales et al., 2020). Meanwhile, Käbisch *et al.* recently used the *Mhr* type strain DSM 25591 to establish a repeatable protocol for antimicrobial susceptibility testing of this bacterium, aiming to promote inter-laboratory standardisation and more targeted use of antimicrobials (Käbisch et al., 2023).

The antibiotic susceptibility by broth micro-dilution method and mismatch amplification mutation assay (MAMA) were tested in 76 *Mhr* isolates from Belgium, Germany, Hungary, Italy and Poland collected between 2019 and 2021 (Klein et al., 2022). Isolates were sensitive to low concentrations of tiamulin, doxycycline, oxytetracycline and florfenicol and to moderate concentrations of enrofloxacin. For the tested macrolides and lincomycin, a bimodal MIC pattern was observed. Interestingly, resistance to macrolides and lincomycin in three of the isolates was not associated with SNP A2066G in the 23S rRNA gene (Klein et al., 2022).

A review of the use of antimicrobials for the treatment of *Mhp* described the resistance mechanisms that have been reported (Maes et al., 2020). Acquired resistance to macrolides and lincosamides in field strains of *Mhp* has been associated with A2058G, A2059G and A2064G (*E. coli* numbering) point mutations in domain V of the 23S rRNA gene. Mutations in domain II of 23S rRNA or in proteins L4 and L22 that have been identified in other *Mycoplasma* species have not been described in *Mhp* to date. Reduced susceptibility to marbofloxacin in *Mhp* was associated with an S80F or an D84N in the ParC QRDR, while reduced susceptibility to flumequine and enrofloxacin was associated with an S80Y substitution in the ParC QRDR, and an extra A83V mutation in GyrA further reduced enrofloxacin susceptibility in one strain. These and two additional mutations were found in the GyrA QRDR: G81A and E87G of Hungarian *Mhp* field isolates (Felde et al., 2018a). These studies suggest that ParC could be the primary target of fluoroquinolones in *Mhp* (Maes et al., 2020). Initially, single mutations observed in strains showed moderately reduced susceptibility, while subsequent double substitutions in the *parC* and *gyrA* genes were associated with higher MIC values (Felde et al., 2018a; Maes et al., 2020). Although strains with increased resistance have been identified, to date, no mechanism(s) of resistance to tetracyclines, aminoglycosides/aminocyclitols, pleuromutilins or amphenicols has been described in *Mhp* (Maes et al., 2020).

MAMAs and high-resolution melting (HRM) analysis designed for the rapid detection of antimicrobial resistance-associated mutations have been designed (Felde et al., 2020). The results of the MAMA and HRM assays matched the results of the MIC susceptibility method for the *Mhp* field isolates tested (Felde et al., 2020). A separate study has developed a MAMA to detect the mutation of the 23S rRNA

gene at nucleotide position 2058 associated with resistance to macrolides and lincomycin (Földi et al., 2021). These molecular assays are convenient and cost-effective and provide information about the antimicrobial susceptibility of *Mhp* to critically important antimicrobials in a significantly shorter time compared to conventional MIC tests (Felde et al., 2020; Földi et al., 2021).

Vaccines

There are many vaccines currently available to protect against *Mhp,* spanning mono-, bi- and tri-valent bacterins, while in China a live-attenuated mycoplasma vaccine based on strain 168L is also used. However, the performance of the current vaccines is not optimal, with none advertising either complete clinical protection or the prevention of colonisation. Housing and management practices may also impact their efficacy, as illustrated in a recent study in Greece, where, despite a 98.3% vaccination rate, almost 50% of pig lungs contained lesions consistent with *Mhp* pneumonia (Lisgara et al., 2022). Most of these vaccines are generally administered intramuscularly, though we know that this may not be the optimal approach either immunologically or practically: in summary, there is room for improvement of the vaccines themselves, the way they are used, and how they are incorporated into wider herd-management practices.

Although the challenge of complete protection by vaccines has yet to be overcome, marked progress has been made. The development of new multi-valent formulations that offer comparable immunity to single-shot vaccines but with practical advantages for the farmer, and welfare advantages for their stock, is a notable advance. Meanwhile, researchers have been working hard to devise and test novel vaccination approaches and new formulations of existing agents.

Current vaccines

The introduction of a new trivalent vaccine against porcine circovirus (PCV) 2a/b and *Mhp* by Zoetis in 2019, under the tradenames Fostera® Gold PCV MH in the USA and Asia/CircoMax® Myco in Europe, spurred a flurry of research studies: as well as inactivated *Mhp,* the inclusion of killed PCV2b offered, for the first time, the potential for cross-protection against the genetically similar PCV2d genotype, which has become the most prevalent strain globally in recent years and seems able to evade immunity induced by vaccines against PCV2a (Huan et al., 2018). Researchers from the parent company provided evidence of long-lived immunity, with a single vaccination at approximately three weeks of age conferring significant protection from challenge six months later (Gracia et al., 2021). Other studies have since tested the value of this vaccine under more diverse field and experimental

conditions. Um *et al.* showed that either one or two immunisations offer good protection against *Mhp* in terms of reduction in infectious load and improved growth characteristics in healthy piglets within a field herd with existing PCV2 and enzootic pneumonia, both in its own right (Um et al., 2021a), and when compared to the bivalent Porcilis® PCV M Hyo (MSD Animal Health) (Um et al., 2021b). Moreover, immunological and growth performance in the field was better following Fostera® Gold immunisation of piglets from a commercial farm compared to vaccination with the bivalent Ingelvac FLEXcombo (Boehringer Ingelheim) and Porcilis® (MSD Animal Health) (S. Yang et al., 2022).

Fostera® Gold is also one of the commercially available vaccines that has proven amenable to needle-free delivery, exhibiting comparable induction of specific antibodies and reduction in pathology when applied via intramuscular injection or by either of two needle-free injection devices to pigs under field conditions (Cho et al., 2022). Similarly, co-administration of a single dose of each of two commercially available PCV and *Mhp* vaccines using an intra-dermal needle-free delivery device conferred complete protection from clinical signs and significantly reduced the pathology associated with co-challenge three weeks later (Lee et al., 2021). A single small trial reported the effective adaptation of an attenuated *Mhp* vaccine licensed in China to aerosol delivery, showing that piglets exposed to the aerosol in a sealed chamber and challenged 42 days later were partially protected from lung lesions, comparable to the protection elicited by intra-pulmonary injection of the same vaccine (Hao et al., 2022a). However, using this method, only one in three piglets exhibited a specific sIgA response in the lung, while all piglets vaccinated via the intra-pulmonary route exhibited sIgA responses (Hao et al., 2022a). By contrast, when Li *et al.* compared the immunological effects of administering a live-attenuated *Mhp* strain (*Mhp* 168), which has been used to control the disease in China, once via intramuscular or intrapulmonary injection or twice intranasally, they found that the intrapulmonary route was significantly better at inducing specific IgA production in the respiratory mucosa (PengCheng et al., 2015).

Numerous trials have added to our knowledge of the performance of more established commercially available vaccines under experimental challenge or field conditions. Among the monovalent products, a single immunisation with the *Mhp* bacterin vaccine Hyogen® (CEVA Santé Animale) at three weeks of age led to improved weight gain and fewer *Mhp*-associated lung lesions at slaughter under field conditions, compared to unvaccinated animals (Kim et al., 2021). Alongside, a field study demonstrated increased production and decreased lung lesions in pigs vaccinated once with Ingelvac MycoFLEX® (Boehringer Ingelheim) at three weeks of age (Pepovich, 2019). Similarly, a single immunisation of the inactivated *Mhp* vaccine Hyogen® was sufficient to protect piglets from

subsequent co-challenge with low- and high-virulence strains of *Mhp* (Michiels et al., 2017a). Lastly, Cvjetković *et al.* conducted a large field study in an Austrian farrow-to-finish farm that compared a single immunisation with Hyogen® at approximately three weeks of age against two immunisations with Stellamune® Mycoplasma (Eli Lilly) at four days and roughly three weeks of age, finding that the former provided significantly superior protection against extension of lung lesions and prevalence of bronchopneumonia at the point of slaughter (Cvjetković et al., 2018). A recent study focused on characterising the immune response to Ingelvac MycoFLEX® along the lifespan of commercially bred piglets reared on farms in which *Mhp* had previously been shown to be circulating: interestingly, despite vaccination at 16 days of age, all piglets (whether born to sows with anti-*Mhp* antibodies or not) exhibited declining levels of antibodies during the course of their lives, returning to baseline from three months; while *ex vivo* recall T cell proliferative and cytokine responses peaked at one month and also then declined, but were present until the end of the experiment at six months (Biebaut et al., 2023).

Studies of bivalent vaccines including *Mhp* have also evidenced a level of efficacy that is relevant to producers. The PCV2 recombinant vector/*Mhp* bacterin vaccine, Circo/MycoGard® (Pharmgate Animal Health) conferred partial protection from dual challenge of piglets 25 days after a single immunisation (Ahn et al., 2021); while Suh *et al.* found that an intradermally administered bivalent vaccine comprising killed recombinant *Mhp* expressing a PCV2a capsid protein also provided partial protection from experimental challenge in piglets vaccinated once and exposed to either single or co-challenge approximately one month later (Suh et al., 2022). Another study compared two commercially available bivalent *Mhp*/PCV vaccines, Porcilis® PCV M Hyo (Intervet) and Suvaxyn® Circo+MH RTU (Zoetis), in a field setting in which both pathogens were present, finding that the two vaccines each conferred significant protection from disease and the associated reduced growth performance of piglets, but that Porcilis® PCV M Hyo offered superior responses under the tested conditions (López-Lorenzo et al., 2021). Similarly, in a field study at a Dutch farrow-to-finish farm with both PCV2 and *Mhp* present in the herd, piglets vaccinated once at three weeks of age with Porcilis® PCV M Hyo exhibited improved protection from both PCV2 and lung lesions at slaughter compared to those vaccinated concurrently with both CircoFLEX® and MycoFLEX®, or their unvaccinated littermates (Kaalberg et al., 2017). These data also supported earlier work in Poland which found that Porcilis® PCV M Hyo was well-tolerated by immunised piglets and significantly protected them from the adverse effects of circulating PCV2 and *Mhp* (Witvliet et al., 2015).

Potentially important insight into the utility of multivalent versus multiple monovalent vaccines has also been generated: Oh *et al.* showed that a trivalent vaccine (3FLEX®, Boehringer Ingelheim Vetmedica) including *Mhp* gave comparable protection against *Mhp* challenge compared to monovalent vaccination with MycoFLEX® 35 days later, though immunity to PRRSV was poorer following multivalent immunisation compared to after administration of monovalent Ingelvac PRRS® MLV (Boehringer Ingelheim) (Oh et al., 2019). However, another recent study suggested that co-immunisation against PCV (Porcilis PCV ID), PRRSV (Porcilis PRRS ID), *Lawsonia intracellularis* (Porcilis Lawsonia ID) and *Mhp* (Porcilis M Hyo ID ONCE) via the intradermal route did not compromise protection from individual pathogen challenge (Horsington et al., 2021); likewise, single- and co-immunisation against *Mhp* (Hyogen®) and PCV2 (Circovac®) induced comparable immune and protective features following challenge with either pathogen (Sibila et al., 2020). In contrast, co-immunisation with Ingelvac® Circoflex® and Ingelvac® Mhyo did not protect against lung lesions to the same level as the bivalent Porcilis® PCV M. Hyo vaccine under field conditions, though overall growth parameters were comparable between these groups (Pagot et al., 2017).

As well as optimal choice of the vaccine itself for the setting, vaccine efficacy at both the individual and herd levels can be impacted by life-stage/age of the animal. Immunisation of sows against *Mhp* may confer a useful level of protection upon their progeny, but results vary: while Arsenakis *et al.* concluded that sow vaccination could be useful, they also found that responses were highly variable between herds (Arsenakis et al., 2019); more recently, De Conti *et al.* showed that the parity of sows was a determining factor in their own vaccine responses and that the immunity also passed to their offspring, suggesting that vaccination efforts should be focused on primiparous sows (De Conti et al., 2022). On the piglet side, Yang *et al.* showed that vaccination against *Mhp* was more effective at reducing pathogen shedding and protecting growth performance when administered at one week of age than at three weeks of age, as part of a multi-pathogen immunisation and challenge experiment (Yang et al., 2020).

Alongside *Mhp, Mhr* is an important pathogen in commercial swine production, but until recently there was not a vaccine available. In 2019, this changed: following the development of a suitable challenge model (Martinson et al., 2018a) and the demonstration of the candidate vaccine's ability to partially protect colostrum-deprived piglets from clinical disease caused by *Mhr* and to decrease the impact of infection on daily weight gain following infection (Martinson et al., 2019, 2018b), the first *Mhr* vaccine was licensed. Brought to market by Boehringer Ingelheim Animal Health under the trade name Ingelvac® MycoMAX, this binary ethyleneimine (BEI)-inactivated bacterin vaccine could help to

markedly decrease the impact of *Mhr* infection on commercial swine farmers; however, widespread testing under field conditions has yet, to our knowledge, to be conducted. Alongside, an ELISA based on the *Mhr* protein p37 has been developed that is able to measure specific antibody responses to this pathogen and discriminate them from responses to closely related mycoplasmas (Bumgardner et al., 2018).

Novel vaccines

Inactivated vaccines

Alongside the commercially available bacterin vaccines against *Mhp,* several studies have reported new inactivated vaccines. For example, in 2019, Matthijs *et al.* published two studies describing their work towards a novel bacterin vaccine against *Mhp,* based on the highly virulent and antigenically distinct F7.2C strain: in the first study, they trialled a range of different formulations, identifying microparticle and oil-in-water formulations as effective at delivering a TLR ligand cocktail designed to enhance cellular immune responses, and successfully using cationic liposomes to deliver a ligand for Mincle (macrophage inducible C-type lectin) that elicited Th17 cells (Matthijs et al., 2019b). The group then took these three most promising candidates into trials of protection, finding that the oil-in-water formulation including TLR 1/2, 7/8 and 9 ligands offered the highest level of clinical protection, coupled with the greatest reduction in pathogen shedding and increased levels of IgA in the lung (Matthijs et al., 2019a). However, none of the vaccines offered complete protection from disease, and it is not yet clear how their performance compares to that of commercially available vaccines, or under field conditions: these promising results warrant further study.

In their initial study, Matthijs *et al.* also reported links between the blood transcriptome of animals in the first day post-vaccination and the magnitude of the later-observed immune responses, finding that activation of transcriptional modules consistent with a strong innate response, followed by their rapid down-regulation, was associated with a protective adaptive response to the vaccine (Matthijs et al., 2019b). Around the same time, Munyaka *et al.* identified differentially expressed genes in the peripheral blood of *Mhp* bacterin-immunised piglets that were also associated with high and low serological responses, including upregulated expression of genes involved in activation of the immune response at two days post-vaccination, followed by their down-regulation at day six (Munyaka et al., 2019). These data together begin to shape our understanding of post-vaccine immune correlates of protection, which could also be relevant when assessing the responses of MPS-resistant swine breeds to infection and vaccination.

A series of studies has described investigations of an oral bacterin vaccine against *Mhp*, comprising a blend of total *Mhp* antigens with nanostructured mesoporous silica, designed to allow slow release in the intestine and thereby promote mucosal immunity; indeed, IgA responses in the respiratory mucosa of slaughter pigs were comparable to those elicited by intramuscular immunisation with a commercial bacterin vaccine, as was the level of protection from lung lesions following experimental challenge, though the oral vaccine did not induce detectable levels of systemic IgG, while – at least in this study – the commercial vaccine did (Mechler-Dreibi et al., 2021). Following on from this work, the oral vaccine was administered to piglets in which it similarly induced specific immune responses, but did not perform as well as the conventional vaccine in terms of immune parameters or protection from challenge after a single immunisation (Ferreira et al., 2023); though emerging evidence suggests that combining this oral vaccine with a commercially available bacterin boost, may enhance the protective response under field conditions (Storino et al., 2023).

Live-attenuated vaccines

Li *et al.* reported the successful generation and characterisation of a passage-attenuated strain of *Mhp* ES-2: after 200 passages, the attenuated strain was non-pathogenic in pigs and had lost over 37 kbp of its genome, including genes involved in alcohol dehydrogenation (which is linked to production of H_2O_2 under anaerobic conditions) and those encoding the LppA family lipoprotein and transcription elongation factor GreA, which are known virulence factors (Z. Li et al., 2021). The immunogenicity of the strain in terms of its ability to protect from pathogenic challenge has yet to be assessed.

Subunit vaccines

Several groups have made marked progress towards the development of a defined subunit vaccine, though many candidates remain at an early stage of development. Using reverse vaccinology, Li *et al.* generated a recombinant subunit vaccine comprising 11 predicted major histocompatibility complex (MHC)I/MHCII-binding epitopes from within an *Mhp* membrane nuclease, reporting strong immunogenicity in mice and piglets (Li et al., 2022). However, it is not yet clear whether the responses detected would protect pigs from challenge. Similarly, de Oliveira *et al.* constructed a chimeric protein comprising *Mhp* antigenic proteins P97R1, P46, P95 and P42, which was recognised by sera from convalescent pigs, and injected it into mice; they found that whether the protein was formulated as an adjuvanted subunit vaccine, or as an inactivated bacterin vectored by *E. coli*, it induced strong systemic IgG responses, with an IgG1 bias (N. R. de Oliveira et al., 2017). More recently, a study in mice reported a strong Th1 cellular response and the generation of specific antibodies following two

immunisations with an adjuvanted chimeric protein incorporating B and T cell epitopes from *Mhp* P46 and P65, and from PRRSV-GP5 and M proteins (Gao et al., 2022).

The *Mhp* protein P97 is the target of two additional novel subunit vaccines. In 2019, Ferriera *et al.* reported interesting preliminary results comparing intranasal and intramuscular administration of a novel *Mhp* P97- *E. coli* heat-labile-enterotoxin-fusion protein, designed to elicit strong mucosal immunity; they found that intranasal administration alone was able to completely protect piglets from lung lesions and colonisation of the respiratory tract following virulent challenge, despite the lack of detectable seroconversion in the blood (Ferreira et al., 2019). At around the same time, Hahn *et al.* were granted a patent in the USA for a vaccine formulation based on recombinant *Mhp* P97 protein, after demonstrating its superior performance compared to a commercially available inactivated *Mhp* vaccine (Hahn et al., 2019). Taken together, this antigen may well form the basis of a successful subunit vaccine in the future.

Vectored vaccines

A single study reported preliminary testing of a recombinant baculovirus-vectored vaccine expressing a chimeric *Mhp* protein comprising the known antigens P97R1, P46 and P42, alongside the PCV2 capsid protein: the vaccine successfully induced both specific antibodies and lymphocyte proliferation in mice and piglets; however, the pig responses were inferior compared to those elicited by a commercially available bacterin vaccine (Tao et al., 2019).

Adjuvants

Adjuvants can have a profound effect on both the magnitude and type of immune response elicited by a vaccine, and therefore its efficacy. Galliher-Beckley *et al.* reported the development of an inexpensive, plant-based, thermostable and antigen-sparing oil-in-water adjuvant, and demonstrated equivalent or superior strength and duration of antibody responses in piglets to inactivated *Mhp* compared to a commercially available *Mhp* bacterin vaccine (Galliher-Beckley et al., 2015). Another plant-based adjuvant, fucoidan, similarly showed promise in increasing antibody responses to *Mhp* antigen in mice (SuYeon and HongGu, 2015), but has yet to be tested in pigs, to our knowledge.

Nanoparticles may also be able to enhance responses to both subunit and whole-inactivated vaccines against *Mhp.* Virginio *et al.* concluded from mouse immunisation studies that mesoporous silica nanoparticles elicited superior humoral and cell-mediated immune responses to an *Mhp* Hsp70 polypeptide domain compared to conventional alum-based adjuvant (Virginio et al., 2017). While,

more recently, combining inactivated *Mhp* with a chitosan-coated nanoemulsion was found to be a promising basis for a dry powder vaccine for nasal vaccination: Canelli *et al.* observed found that a single intra-nasal immunisation with the novel vaccine induced significantly higher humoral and cell-mediated immune responses – most notably including high levels of IgA in the respiratory mucosa – to *Mhp* compared to intra-muscular injection with an unspecified commercially available bacterin vaccine (Canelli et al., 2023).

Rational testing of promising novel vaccine candidates in combination with appropriate conventional or novel adjuvants is a key strategy to advance the field of vaccine research.

Conclusions

Mhp remains a highly prevalent and economically important pathogen of swine populations worldwide, and the challenges inherent in its study (including the difficulty of culturing mycoplasmas and the chronic nature of *Mhp* infections) continue to limit our knowledge of its biology, transmission, and other important factors. However, recent studies have greatly expanded our understanding of *Mhp* despite these limitations, including the identification of many previously unknown factors in the biology, pathology and immunology of this disease. Over the past ten years, researchers have defined new methods for typing and diagnosing *Mhp*, which have facilitated higher-resolution studies of this pathogen's evolution and opened new avenues for on-farm surveillance, and the continuing generation of novel vaccine candidates has provided a firm basis for the validation and field testing of new control measures. Altogether, the progress made over the past decade in understanding and managing swine mycoplasma infections has been considerable and has placed a solid foundation for future research to address the many questions that remain.

Alongside these advances, the potential roles of *Mfl*, *Mhr* and *Mhs* – alone and/or in co-infection with *Mhp* – have also been studied, and new strategies are now available for their detection, molecular classification, and control. The same is true for the haemoplasmas, though the recent identification of the putatively pathogenic *C.* M. haemosuis has raised new questions that will require many studies to answer over the coming years.

Future research priorities

Based on the available literature, expert opinion, and previously published reviews (DISCONTOOLS, 2016; Maes et al., 2021, 2018; Sargeant et al., 2019), we suggest that the following areas of research into mycoplasmas affecting swine should be considered high priority:

Biology of the pathogens

- *Genome phylogeny studies, including data on disease association, to better understand the global distribution and evolution of swine mycoplasmas*
- *Deeper understanding of relationships between swine mycoplasmas and their associations with clinical disease and the wider respiratory microbiome*

Diagnosis

- *Further work to optimise sensitive, specific, affordable methods of surveillance for early detection of infection within populations*
- *Development of DIVA-capable serodiagnostics*
- *Continuing development of rapid and easy-to-use pen-side diagnostics*
- *Improved capabilities for sampling live animals*

Pathogenesis

- *Improved molecular tools (mutagenesis methods) to dissect and confirm the role of putative virulence factors as future candidates for vaccines or surveillance*
- *Continuing characterisation of the roles of glycerol and myoinositol metabolism in* Mhp *virulence*
- *Clarification of* Mhp *strain-specific differences in disease severity*

Immunology

- *Further work to understand and learn how to mitigate the immune-dysregulatory effects of* Mhp*, especially in the challenge of mixed infections*
- *Combinatorial/cross-breeding studies to maximise the benefits of immune-resistance traits identified in different breeds/studies*
- *Assessment of the* in vivo *significance of innate immune findings made* in vitro*, for example, the degradation of NETs/METs and the use of derived nucleotides for replication*

- *Transition away from* in vitro *studies in the 3D4/21 PAM cell line due to documented differences related to gene insertion and immortalisation*
- *Characterisation of the relative importance of maternally derived antibodies and cell-mediated immunity in protecting offspring*

Epidemiology

- *Increasing focus on* Mhp *epidemiology and control in Africa*
- *Work to validate existing empirical assumptions around gilt acclimation methods aimed at reducing shedding in parity-1 litters*
- *Further work to fully understand infection dynamics of Mhp, Mhr and Mhs in the suckling period, and consequent susceptibility of piglets in the immediate post-weaning periods*
- *Updating of old research to describe risk factors for aerosol transmission of Mhp between farms*
- *Continued integration of molecular biology studies (e.g., genome sequencing and molecular typing) and comparison against the PubMLST database to monitor the evolution and geographic spread of swine mycoplasmas*
- *Computational modelling of risk factors and estimated transmission networks*
- *Investigation of the prevalence of* C. *M. haemosuis under different geographical and farm management conditions*

Control

- *Work to assess the value of or need for updating isolates included in existing commercial bacterins*
- *Development of alternatives to antibiotics for the treatment of infected animals*
- *Implementation of effective surveillance and monitoring schemes in wild boar populations*
- *Development of improved vaccines with greater protective efficacy and/or ability to inhibit disease transmission within swine herds*
- *More work to understand the effect of sow vaccination on reduction of shedding/transmission in the suckling period*
- *More work to identify adjuvants that deliver optimised mucosal immunity*
- *Continued studies of the potential generation of* Mhp*-resistant pig breeds and related immunogenetic patterns*
- *Standardisation of vaccine validation study protocols (e.g., housing of animals, inoculation route, etc.); inclusion of best-performing commercial vaccine as the gold-standard control*

- *Determination of duration of immunity offered by new trivalent* Mhp/PCV2(a/b) *vaccine under field conditions*

Mycoplasmas affecting poultry

Introduction

Mycoplasma gallisepticum (*M. gal*) and *Mycoplasma synoviae* (*M. syn*) are the most important pathogenic mycoplasmas of poultry, causing acute or chronic respiratory disease and substantial economic losses in poultry farming systems globally (reviewed in (Chaidez-Ibarra et al., 2022; Yadav et al., 2022b)). The most economically important host animals of these highly transmissible pathogens are chickens and turkeys, but a variety of wild birds are also susceptible to various strains (e.g., the widespread conjunctivitis caused by *M. gal* in American house finches (*Haemorhous mexicanus*)) (Weitzman et al., 2020).

M. gal has been an established global pathogen for decades, and its clinical presentations in domestic poultry reduce meat and egg production, increase embryo morbidity, and require expensive control measures that can contribute to overuse of antibiotics and establishment of antimicrobial resistance (De la Cruz et al., 2020; Giram et al., 2022; WOAH, 2022b). Studies of its economic impact are rare, but annual losses due to *M. gal* in the USA alone have been estimated at 118-150 million USD (~241-307 million USD adjusted for inflation in 2023) (Evans et al., 2005; Patterson, 1994).

Similar to *M. gal*, *M. syn* infection is associated with acute or chronic respiratory disease, but its tropism for joints also causes arthritis and lameness in infected birds (WOAH, 2022b). *M. syn* has historically been considered a less significant pathogen compared to *M. gal*, but the increasing occurrence of *M. syn*-associated synovitis and eggshell apex abnormalities (which significantly reduce the strength of eggshells produced by infected birds) has attracted greater attention from researchers and policymakers over the past 15 years (Feberwee et al., 2009; Jeon et al., 2014).

Both *M. gal* and *M. syn* are highly prevalent in poultry populations across the world, and disease surveillance and management programmes are lacking in many countries. Commercial vaccines against *M. gal* have been available for many years, and vaccines against *M. syn* have more recently become available ((European Medicines Agency, 2021); reviewed in (Mugunthan et al., 2023)). However, control of these pathogens is complicated by many factors including the inconsistent protective efficacy of these vaccines, the prevalence of chronic disease among *M. gal*/*M. syn*-infected poultry, and a rising trend of antimicrobial resistance (reviewed in (Giram et al., 2022; Mugunthan et al., 2023; Yadav et al., 2022b)).

This section will cover some of the many papers that have been published on the biology, epidemiology, and control of *M. gal* and *M. syn* over the past ten years, highlighting the important advances that have been made in solidifying our understanding of these critical poultry pathogens and reducing their economic impact.

Literature review

Biology of the pathogens

The basic biology of *M. gal* and *M. syn* is similar to the other mycoplasmas described above, with small genome sizes (~820 to 990 kbp in both species, depending on the strain) that belie their molecular complexity and pathogenicity in poultry (Leigh et al., 2019; Papazisi et al., 2003; Xu et al., 2020b; Zhu et al., 2018). Unlike *M. bovis*, numerous vaccines against *M. gal* and *M. syn* are commercially available, including the ts-11, 6/85 and F-strain vaccine strains for *M. gal* and the MS-H and MS1 strains for *M. syn* (reviewed in (Kaboudi and Jbenyeni, 2019; Mugunthan et al., 2023)). The widespread uptake of these vaccines on poultry farms across the world has made molecular typing a particularly important tool for differentiating *M. gal* and *M. syn* field isolates from vaccine strains (Couto et al., 2016), as well as tracking their geographical spread, evolution and molecular diversity. This section will primarily discuss the progress that has been made over the past decade in molecular typing/phylogenetic methods for *M. gal* and *M. syn*, emphasising the application of new sequencing technologies to the collection and comparison of genomic data from these species.

Molecular typing and genome sequencing

Mycoplasma gallisepticum

The *mgc2* gene, which encodes a virulence factor involved in bacterial motility (Indikova et al., 2014), was recently validated as a reliable phylogenetic marker (De la Cruz et al., 2020) and has been widely used for characterisation and differentiation of *M. gal* isolates. Notably, Matucci *et al.* recently reported their identification of an atypical *M. gal* strain, isolated from Italian turkeys, with an *mgc2* variant that was unable to be sequenced by conventional endpoint PCR primers and required an alternative PCR protocol (Matucci et al., 2023). The variable cytadhesin gene *pvpA* has also been used for molecular characterisation of *M. gal* (Boguslavsky et al., 2000; Manimaran et al., 2021), and

Hashemi *et al.* combined this technique with high-resolution melting curve analysis for a phylogenetic study of Iranian *M. gal* isolates (Hashemi et al., 2018).

Other recent studies have reported that at least two partial *M. gal* genes (e.g., the *gapA* and *mgc2* genes) are needed to satisfactorily differentiate *M. gal* isolates (Limsatanun et al., 2022). One response has been to supplement or replace these single gene-targeted sequencing methods with MLST, which displays generally higher discriminative power. Bekő *et al.* developed an MLST assay based on six housekeeping genes, reporting a high level of discrimination and identifying 57 separate sequence types from 130 *M. gal* strains (Bekő et al., 2019b). This method was later used in a study of Italian *M. gal* isolates similar to the vaccine strains 6/85 and ts-11 – here, the authors found that *mgc2* sequencing was only able to identify 6/85-like strains, while MLST also differentiated ts-11-like strains at a higher resolution (Matucci et al., 2020). Ghanem *et al.* developed a core genome MLST (cgMLST) workflow for *M. gal*, using 425 conserved genes to differentiate isolates based on whole-genome sequencing data; initial validation on a set of isolates from different continents demonstrated high-resolution differentiation and good agreement with epidemiological data (Ghanem et al., 2018). In Iran, a smaller-scale gene-targeted sequencing workflow was developed for *M. gal* strain differentiation based on the complete *mgc2* and *pvpA* gene sequences, with the authors reporting higher discriminatory power compared to typing based on partial gene sequences (Bashashati and Banani, 2020).

As the above studies indicate, the continuing generation of complete sequence data is vital for tracking the epidemiology and evolution of *M. gal*, and the advancement of next- and third-generation sequencing technologies has facilitated a growing number of reports of complete genome sequences. Leigh *et al.* used Illumina and MinION Nanopore to sequence two derivatives of the original F-strain isolate (a laboratory-maintained F99 strain and the commercial AviPro vaccine strain) (Leigh et al., 2019) and later published the sequences of three isolates (K4043, K4421A and K5234) that are extremely similar to the 6/85 vaccine strain (Leigh and Evans, 2022).

Mycoplasma synoviae

Molecular typing tools for *M. syn* have also seen significant developments over the past decade. The first target identified for molecular typing in this species was the variable lipoprotein and haemagglutinin A (*vlhA*) gene, which is part of a gene family contained in a single region of the *M. syn* genome (Béjaoui Khiari et al., 2010). While most of the *vlhA* gene is semi- or highly variable, playing an important role in *M. syn* antigenic variation by altering the immunogenic surface haemagglutinin

(Noormohammadi et al., 2000), an upstream segment in the gene's conserved region is present as a single copy in the genome and can be sequenced to discriminate and type *M. syn* strains (El-Gazzar et al., 2012). *vlhA* sequencing broadly replaced earlier, more labour-intensive methods of *M. syn* molecular typing (Dijkman et al., 2016), and recent studies have characterised *M. syn* strains via *vlhA* sequence comparison in several countries, including Egypt (Tawfik et al., 2018), Pakistan (Rasool et al., 2018), India (Rajkumar et al., 2020), and China (ShiKai et al., 2017b). This latter study reported no significant link between pathogenicity and the analysed 5'-end of the *vlhA* gene, highlighting the difficulties that remain in predicting *M. syn* virulence based on sequence data (ShiKai et al., 2017b).

While *vlhA* typing is a relatively simple and reliable method for gaining phylogenetic information on *M. syn* isolates, *vlhA* partial sequences can vary substantially even between closely related strains, making it difficult to determine the genetic closeness of two isolates based on this method alone (Dijkman et al., 2014; El-Gazzar et al., 2016). Therefore, as for *M. gal*, MLST has also been applied as a more discriminative typing method for *M. syn*. Three independent MLST workflows for *M. syn* were published in 2016. Cizelj *et al.* based their method on six loci including three polymorphic genes (5'-*vlhA, cysP* and *nanH*), reporting good discrimination and separation of genotypes into clusters of epidemiologically linked strains (Cizelj et al., 2015); El-Gazzar *et al.* combined an MLST workflow with *vlhA* typing, using internal sequences from seven housekeeping genes to differentiate isolates at higher resolution (El-Gazzar et al., 2016); and Dijkman *et al.* selected five target genes to monitor for SNPs and reported higher discriminatory power compared to *vlhA* typing (Dijkman et al., 2016). MLVA has also been used for *M. syn* molecular typing, displaying similar discriminative power as a cost-effective alternative to MLST in a recent comparative study of *M. syn* isolates from 15 countries (Kreizinger et al., 2018).

Alongside their cgMLST method for *M. gal*, Ghanem *et al.* developed a similar workflow for *M. syn*, using 302 core genes to type whole-genome sequences from geographically disparate isolates and reporting high discriminatory power and agreement with epidemiological studies (Ghanem and El-Gazzar, 2018). Meanwhile, a standard MLST method (based on seven housekeeping genes) was employed in a molecular analysis of *M. syn* strains in China, finding that all analysed isolates belonged to a new phylogenetic group independent of previously reported strains (X. Zhang et al., 2021b).

Finally, several new *M. syn* genome sequences have been published over the past decade, including strains HN01 and 5-9 from China (Xu et al., 2020b; X. Zhang et al., 2021a). Zhu *et al.* used Illumina and MinION Nanopore methods to obtain the complete genome sequence of the widely used MS-H live

attenuated vaccine strain and compared it against an older American strain (WVU-1853) and a more recent Brazilian strain (MS53), identifying unique genetic features in MS-H that could be used for new DIVA diagnostics (Zhu et al., 2018).

Proteomics and gene characterisation

Mycoplasma gallisepticum

A protocol was recently published for targeted mutagenesis in *M. gal* via its endogenous CRISPR/Cas system, editing the *ksgA* gene to produce a distinct phenotype by abrogating resistance to the aminoglycoside kasugamycin (Mahdizadeh et al., 2020a). These authors later expanded this system to include simultaneous targeting of the *mnuA* nuclease gene (Klose et al., 2022b). This workflow could provide a significant new opportunity to identify the functions of *M. gal* gene products, many of which remain uncharacterised.

Mycoplasma synoviae

There have not been any notable advances published in this area since 2015.

Diagnosis

As with other pathogenic mycoplasmas, isolation and culturing is the gold standard for diagnosing infections with *M. gal* or *M. syn*; however, the time and effort involved in culturing mycoplasmas makes this impractical for rapid diagnosis (Gondal et al., 2015; Khalifa et al., 2013) and can lead to false negative results (Ammar et al., 2016a; Bukte and Gandge, 2018; Muhammad et al., 2018; Qasem et al., 2015). Instead, serological tests (e.g., serum plate agglutination or ELISA) are commonly used to screen flocks for infection, and positive results are confirmed via molecular assays (Yadav et al., 2021). Multiple diagnostics can also be applied to provide a clear picture of vaccine behaviour within a poultry population – a study of the MS-H strain within Italian broiler breeders, for instance, used serology and PCR to evaluate the transmission patterns of this vaccine at different production stages (Moronato et al., 2018).

DNA diagnostics

As co-infections between *M. gal* and *M. syn* are common, diagnostic assays able to detect and discriminate between these pathogens are useful for poultry premises in regions with high prevalence of both pathogens. One reported duplex qPCR assay used TaqMan minor groove binder probes targeting the *mgc2* and *vlhA* genes of *M. gal* and *M. syn*, respectively; the authors reported that the use of these probes simplified testing and improved specificity compared to similar assays (Ehtisham-ul-Haque et al., 2015). Meanwhile, Yadav *et al.* developed a duplex assay targeting the 16S-23S intergenic spacer region of *M. gal* and *vlhA* in *M. syn*, reporting ~94% diagnostic specificity and 100% specificity compared to single-species PCR (Yadav et al., 2022a).

Li *et al.* used duplex PCR to discriminate *M. syn* from non-mycoplasma respiratory pathogens (e.g., avian reovirus) (Li et al., 2015). Croville *et al.* took this a step further, publishing proof-of-concept for detecting 15 avian respiratory pathogens (including *M. gal* and *M. syn* alongside other common bacteria and viruses) in simultaneous qPCR assays on a commercial Biomark high-throughput PCR nanofluidic system (Croville et al., 2018).

PCR has also been combined with high-resolution melt curve analysis (Ghorashi et al., 2015) or restriction digestion (Zakeri and Pourbakhsh, 2017) to differentiate between vaccine and field mycoplasma strains, which is critical for appropriately monitoring transmission patterns and evaluating the efficacy of vaccine programmes. Two assays were recently developed for discriminating field *M. syn* from the MS-H vaccine strain: a qPCR test to detect differences in the *obg* gene (Dijkman et al., 2017) and a combination of nested PCR and high-resolution melting curve analysis targeting an adenine deletion in the vaccine's *oppF-1* gene (Zhu et al., 2017). Ricketts *et al.* published a combination of PCRs for discriminating *M. gal* vaccine strain ts-11 from the virulent R_{low} field strain via mutations in *vlhA* and the hypothetical protein gene *mg0359* (Ricketts et al., 2017).

As discussed in other sections, isothermal DNA amplification-based diagnostics promote the development of field-capable assays by removing the need for a thermocycler and (to a varying extent) experienced laboratory personnel to run the assay. Kuo *et al.* evaluated GeneReach's commercial POCKIT *M. synoviae* detection kit, an insulated isothermal PCR assay that targets the *vlhA* gene of *M. syn*, on synovial fluid and respiratory tract swab samples from Taiwanese poultry farms, reporting ~98% agreement with reference qPCR and no cross-reaction with *M. gal* or other economically significant poultry pathogens (Kuo et al., 2017). The popular LAMP approach has also been applied to

poultry mycoplasmas; Kursa *et al.* developed a *vlhA*-targeted LAMP assay and tested it on Polish field isolates of *M. syn*, reporting 1,000-fold higher sensitivity versus conventional PCR after 1 hour in a 63°C water bath (Kursa et al., 2015). LAMP assays for *M. gal* diagnosis have targeted the *mgc2* (Ehtisham-ul-Haque et al., 2017) or *pdhA* (FanQing et al., 2015) genes, with results generally indicating high speed and sensitivity.

Alongside LAMP, a polymerase spiral reaction has been developed for *M. syn* diagnosis, reportedly providing 100-fold higher sensitivity over PCR and allowing visual readout of assay results (QianQian et al., 2019); meanwhile, Xia *et al.* developed a recombinase-aided amplification assay for *M. syn*, combining it with a lateral flow dipstick for visual readout – the authors reported an agreement rate of ~95% with traditional PCR when tested on clinical samples, with a total assay time of 25 minutes (Xia et al., 2022).

As the above studies indicate, most molecular diagnostics for *M. syn* have targeted the conserved *vlhA* gene, but other targets of interest have recently been identified within the *M. syn* genome. Xu *et al.* identified and developed SYBR green-based qPCR assays against two conserved *M. syn* genes (dubbed VY93_RS02250 and _RS02300), reporting 100% sensitivity and specificity in testing clinical samples (Xu et al., 2021).

Finally, Bekő *et al.* developed rapid MAMAs to identify antimicrobial resistance-associated mutations in *M. syn* strains, reporting high coincidence with traditional *in vitro* sensitivity tests (Bekő et al., 2020b). MAMAs have also been applied to differentiate between vaccine and field strains of *M. gal* (Sulyok et al., 2018) and *M. syn* (Kreizinger et al., 2015).

Serological diagnostics

The simplicity and ease-of-use of serological assays make them particularly suitable for assessing herd-level prevalence before moving to more sensitive, and more complicated, molecular assays. Serum plate agglutination assays have been widely reported in the literature, but their false positive rate is often high compared to ELISA (Ezatkhah et al., 2015). A recent comparison of commercial serodiagnostics (one rapid plate agglutination test and three ELISAs each against *M. gal*, *M. syn*, and *M. gal*+*M. syn*) found that the specificity of the agglutination test was too low for reliable discrimination of *M. gal*/*M. syn* from other poultry mycoplasmas (Feberwee et al., 2020).

Some in-house serum plate agglutination tests have been reported to out-perform specific ELISAs (Manimaran et al., 2020; Wanasawaeng et al., 2015), and in-house serological assays against regional *M. gal*/*M. syn* isolates may perform comparably to commercial kits at a lower cost (Rasool et al., 2017). In Egypt, for instance, Elyazeed *et al.* compared commercial ELISAs and PCR against in-house ELISA kits and a lateral flow assay targeting the local *M. gal* strain EGY1-2017; they observed a strong correlation between the compared ELISAs, though the lateral flow assay fared worse relative to PCR (~78% sensitivity and 92% specificity) (Elyazeed et al., 2020).

The reliability and relative simplicity of the ELISA platform has facilitated the development of several new tests for *M. gal*/*M. syn* diagnosis over the past ten years. An indirect ELISA based on the recombinant C-terminus of the *M. syn* OppF protein was reported to discriminate between antibody responses against the MS-H vaccine and its parental Australian field strain 86079/7NS, owing to a stable frameshift mutation in the vaccine's *oppF* gene (Kordafshari et al., 2020, 2019). Later, Yadav *et al.* published a whole cell-based indirect ELISA and a dot blot assay for the detection of *M. gal* and *M. syn* antibodies, reporting superior performance by the ELISA (Yadav et al., 2021).

In addition to traditional antibody-based ELISAs, nucleic acid aptamers are increasingly popular antibody detection reagents, as they are produced chemically and are more stable and modifiable compared to monoclonal antibodies (Thiviyanathan and Gorenstein, 2012). A single-stranded DNA aptamer (Apt-236) that binds the *M. gal* PvpA protein was recently used to develop an indirect blocking enzyme-linked aptamer assay, with the study authors reporting an overall coincidence rate of ~92% with a commercial ELISA from IDEXX (Fu et al., 2021).

Sampling strategies and other developments

As new diagnostics continue to be developed, it is also important to develop new sampling techniques and to evaluate existing ones. For instance, Ball *et al.* recently reported that plastic swabs performed significantly better than wooden swabs in the collection of oropharyngeal samples for mycoplasma culture or molecular analysis (Ball et al., 2020), and Assen *et al.* validated molecular analysis of poultry dust particles for monitoring *M. gal* and *M. syn* vaccine uptake and pathogen status (Assen et al., 2022).

Finally, Lorenc *et al.* developed an optical technique for determining whether eggshells were produced by *M. syn*-infected hens based on changes in the shells' transmittance spectra (Lorenc et al., 2019).

This group then published proof-of-concept for using reflected light spectrometry combined with a machine learning algorithm to make this determination, reporting F-scores of 95% for white eggshells and 85% for brown shells (Pakuła et al., 2021). Later, these authors expanded this technique using a multispectral portable fibre-optic reflectometer and signal processing patch; this system performed worse for white but better for brown shells (Pakuła et al., 2022).

Pathogenesis

M. gal has been recognised for many years as an economically important pathogen of domestic poultry, causing chronic respiratory disease and infectious sinusitis associated with increased mortality and reduced weight gain, egg production and egg hatchability (reviewed in (Mugunthan et al., 2023)). *M. gal* infection also disturbs energy metabolism in the lungs, where reduced ATPase activity has been reported in association with cell damage, inflammation and oxidative stress (Ishfaq et al., 2020b), and induces pathological changes in immune organs, including cellular abnormalities, apoptosis and increased oxidative stress in spleen and thymus cells (Hu et al., 2021).

The immune dysfunction induced by *M. gal* infection increases the risk of co-infections with other pathogens, and numerous studies have reported more significant *M. gal*-associated pathologies in co-infected animals. *M. gal*-induced clinical disease was more severe in house finches (*Haemorhous mexicanus*) chronically infected with *Plasmodium* (Dhondt et al., 2017b) or coccidia (Weitzman et al., 2020), for instance; prior *M. gal* infection also reduced antibody titres produced in response to Newcastle disease virus vaccination, and this immune response was even lower in birds co-infected with *E. coli* (Awad et al., 2019). Sid *et al.* studied co-infection with *M. gal* and influenza A virus (IAV) in chicken tracheal organ cultures (TOC), reporting that IAV infection promotes bacterial growth while *M. gal* infection may promote or inhibit IAV replication depending on the timing of bacteria-host interactions (Sid et al., 2016). A later *in vivo* study by Canter *et al.* added to this complex picture, reporting that chickens were significantly more likely to recover from virulent *M. gal* strain R_{low} infection if they had previously been infected with the low-pathogenic IAV H3N8 (Canter et al., 2020). Rüger *et al.* used *in vitro* chicken TOCs to analyse co-infection with *M. gal* and avian metapneumovirus (AMPV), another widespread respiratory pathogen of poultry (Rüger et al., 2021). This study revealed an interesting pattern of competition between the two pathogens wherein pre-infection with AMPV (followed by *M. gal*) enhanced viral replication and innate immunity, while switching the order of infection reduced bacterial attachment, replication, and pathology in poultry (Rüger et al., 2021).

M. syn also causes respiratory disease but infects the synovial membranes as well, leading to lameness alongside similar or slightly lower levels of mortality, egg abnormalities and decreased egg production ((Kursa et al., 2019a); reviewed in (Yadav et al., 2022b)). Atypical presentations such as meningeal vasculitis and encephalitis have also been reported (reviewed in (Rosales et al., 2017)). Like *M. gal*, *M. syn*-associated immune dysregulation in infected animals increases the odds of debilitating co-infections (Giram et al., 2022; Masukagami et al., 2018; Reck et al., 2019; Zhang et al., 2020). A report from Argentina indicated that *M. syn* may also be capable of infecting erythrocytes, thereby promoting chronic infection and allowing bacterial avoidance of antibiotic treatments (Uriarte et al., 2015).

The mechanisms underlying mycoplasma tropism and pathogenesis are complex, involving multifaceted host-pathogen crosstalk and adaptive bacterial alterations at the proteomic, genomic and metabolomic levels (Matyushkina et al., 2016). Meanwhile, "antagonistic coevolution" (reciprocal adaptations in pathogenic mycoplasmas and their host animals) leads to the continuous generation of new bacterial strains and different patterns of host-pathogen interaction (Bonneaud et al., 2018). Identifying the nature of these changes, the cellular and bacterial factors involved, and their impact on virulence is critical for a deeper understanding of *M. gal* and *M. syn* biology and potential routes of control, treatment and vaccination. This section will discuss some of the many advances made over the past decade in these fields.

In vivo *and* ex vivo *studies*

Mycoplasma gallisepticum

M. gal causes chronic respiratory disease by adhering to and subsequently damaging the cilia and mucosa of tracheal epithelial cells (TECs) (Rüger et al., 2022). Unsurprisingly, this damage is associated with significant changes in gene expression: RNA-seq studies of tracheal cells from experimentally infected chickens have identified thousands of DEGs, including many associated with the cell cycle and/or immune defence (e.g., TLRs and the JAK/STAT and MAPK signalling pathways) and mechanical repair (e.g., the ERK-MLCK pathway that regulates tight junction proteins in the trachea) (Beaudet et al., 2017; C. Wu et al., 2022). Prior vaccination has been found to alter this response; chickens vaccinated with the live attenuated vaccine candidate Vaxsafe MG (strain ts-304) and challenged with the virulent strain Ap3AS, for instance, displayed significantly less dysregulation of transcriptional pathways involved in inflammation, cell replication, and mechanical repair compared to unvaccinated birds (Kulappu Arachchige et al., 2020).

The damage caused in the *M. gal*-infected trachea is accompanied by an accumulation of leucocytes in the tracheal submucosa, where Majumder *et al.* found that the interaction of mycoplasma lipid-associated membrane proteins with TLR2 on TECs induced NF-κB-dependent expression of pro-inflammatory cytokines and leucocyte chemoattractants within 90 minutes of infection (Majumder et al., 2014). *M. gal* infection has also been reported to damage the chicken thymus by activating the TLR2/MYD88/NF-κB signalling pathway and the NLRP3 inflammasome, leading to inflammation and immune damage (Chen et al., 2020). Interestingly, pathological changes to the respiratory and gut microbiomes have also been observed in *M. gal*-infected poultry, causing reduced pulmonary immune responses and increased sensitivity to secondary infection by *E. coli* or *Serratia marcescens* (Jian Wang et al., 2021b, 2021a; Wang et al., 2020). The authors of these studies reported several effective countermeasures (including supplementation with the probiotic *Lactobacillus salivarius* and treatment with TLR2 ligand or IL-17) to promote *M. gal* clearance in the lungs, reduce pulmonary damage and restore immunological function (Jian Wang et al., 2021b, 2021a). A link between microbiome and *M. gal* virulence was also observed in house finches, where *M. gal*-induced conjunctivitis is common; disrupting the ocular microbiome with cefazolin (to which mycoplasmas are naturally resistant) increased *M. gal* sialidase enzyme and cytadherence activity in experimentally infected finches, associated with significantly more severe conjunctival inflammation (Thomason et al., 2017). As next-generation sequencing-based protocols are continuing to improve the efficiency of microbiomics assays, large-scale characterisation of these bacterial populations in poultry (e.g., the recent profiling of the upper respiratory microbiome in turkeys (Kursa et al., 2021)) may be a valuable avenue for dissecting host-pathogen interactions and microbiome-associated virulence factors.

Like most pathogenic mycoplasmas, *M. gal* expresses many proteins that have yet to be characterised, and many of the mechanisms underlying its virulence are therefore unknown (Butenko et al., 2017; Tseng et al., 2017a). The identification of new virulence factors is critical to our understanding of *M. gal* biology and to the development of effective countermeasures; this is particularly important in light of the upward trend in virulence observed in *M. gal* isolates over the past 20 years, which has been attributed to adaptations in immune manipulation in response to growing host resistance (Bonneaud et al., 2020; Tardy et al., 2019). Many studies published over the past ten years have therefore aimed to pinpoint and characterise the precise functionality of *M. gal* gene products. The conserved bacterial protein SpxA is one recently described putative virulence factor – in their study of phase transition in *M. gal* during host cell invasion, Matyushkina *et al.* reported that SpxA is a critical regulator of this

process, modulating the expression of key factors in metabolism, translation and adhesion to facilitate adaptation to the host environment (Matyushkina et al., 2016).

Studies of the *M. gal* surface proteome have reported functions for several previously unknown factors involved in adhesion and host-cell pathogen interactions. MGA_0676, formerly reported as a putative lipoprotein but otherwise uncharacterised, was identified as a membrane-localised calcium-dependent cytotoxic nuclease that translocates to the nucleus of infected cells and induces apoptosis (Xu et al., 2015). The mechanism underlying this activity was later studied in detail in DF-1 cells, revealing an interaction between MGA_0676's staphylococcal nuclease region and the Thif domain of NAE (NEDD8-activating enzyme E1 regulatory subunit) that led specifically to NF-κB-mediated apoptosis (Peng et al., 2018). *M. gal*-associated apoptosis has also been reported in chicken PBMCs, mediated by adherence of the *M. gal* membrane protein and protective antigen GroEL (heat shock protein 60) to annexin A2 on the surface of these cells (Tan et al., 2015; Yu et al., 2019). Finally, the *M. gal* membrane proteins GapA and CrmA are known to play important roles in cell adhesion and motility (May et al., 2006), and a recent study in *ex vivo* chicken TOC showed that both proteins are virulence factors that promote adherence, colonisation and tracheal lesion development (Rüger et al., 2022).

Alongside these targeted studies of individual proteins, high-throughput screening technologies have been applied to search for critical proteins within the entire *M. gal* genome. Ron *et al.* applied *in vivo* induced antigen technology to identify 13 putative virulence factors in the R_{low} strain (five previously known and eight newly identified); the immunogenicity and expression levels of these proteins were then validated in experimentally infected chickens (Ron et al., 2015). Tseng *et al.* conducted signature-tagged transposon mutagenesis followed by experimental infection of chickens to identify *M. gal* virulence factors, reporting five new specific mutants (three with insertions in the MGA_0220/$oppD_1$ gene and two in hypothetical genes) with reduced persistence in the respiratory tract (Tseng et al., 2017a). This group then combined metabolomic profiling with bioinformatics analysis to classify MGA_0220 as a likely peptide transporter, while MGA_1102 (one of the two previously identified hypothetical genes) displayed properties consistent with a membrane-associated peptidase (Masukagami et al., 2018). Wang *et al.* used a similar transposon mutagenesis system to pinpoint ten genes associated with biofilm formation, which is thought to promote *M. gal* maintenance in commercial flocks (Chen et al., 2012; Wang et al., 2017).

Finally, the 43 members of the variable lipoprotein hemagglutinin A (*vlhA*) gene family have long been thought to play important roles in *M. gal* immune evasion and pathogenesis (Noormohammadi, 2007), and a recent comparison of *vlhA* expression in *M. gal* strains of varying virulence (including R_{low} and the vaccine strains GT5 and Mg7) supported this – Pflaum *et al.* found evidence for a non-random, temporally regulated progression of *vlhA* gene expression during the first 48 hours of *M. gal* infection in chickens, suggesting that *vlhA* phase variation is an adaptive response to changes in the tracheal environment during colonisation (Pflaum et al., 2018, 2016). An *in silico* modelling study has also been performed on the vlhA proteins, predicting their 3D structures, subcellular localisation and potential functional characteristics via combined application of several publicly available modelling tools (Mugunthan and Harish, 2022).

Mycoplasma synoviae

M. syn emerged as an economically significant pathogen of domestic birds decades after *M. gal*, and relatively fewer studies of its pathogenesis have been published over the past decade compared to *M. gal*. However, the growing prevalence of *M. syn* has necessitated a deeper understanding of its functional proteome and host-pathogen interactions (reviewed in (Kaboudi and Jbenyeni, 2019; Mugunthan et al., 2023; Yadav et al., 2022b)). The complexity of mycoplasma virulence was highlighted in a recent comparison of the whole genome sequences of the widely used MS-H vaccine strain and its parent strain (86079/7NS) – only 32 SNPs were identified in the MS-H genome, with 11 predicted to alter the secondary structure of associated proteins (Ling et al., 2019).

M. syn infection significantly alters gene expression in the host animal – a recent transcriptomic study of chicken tracheal and spleen cells identified 861 and 753 DEGs, respectively, between control vs. *M. syn*-infected birds, with most of these genes relating to cell metabolism and/or immune pathways (Chen et al., 2022); similar findings were previously reported in chicken chondrocytes as well (Cizelj et al., 2016). The immune response to *M. gal* and *M. syn* infections is discussed in more detail in the Immunology section.

Liu *et al.* specifically investigated the mechanisms by which *M. syn* induces synovial infection and arthritis, analysing the transcriptome and proteome of cultured primary chicken synovial fibroblasts after *M. syn* infection (R. Liu et al., 2020). The authors identified 149 genes that were differentially regulated at both the mRNA and protein levels, including factors involved in proliferation, inflammation, angiogenesis and tissue remodelling (R. Liu et al., 2020). One of these proliferation-associated factors was serum amyloid A, which was shown in a subsequent study to accelerate the

cell cycle and promote replication in chicken synovial fibroblasts (R. Liu et al., 2021). Future studies will be needed to clarify the role of increased fibroblast proliferation in *M. syn*-associated synovitis. Pathological infiltration of immune cells is also associated with *M. syn* synovitis, and interactions between *M. syn* and synovial sheath cells have been reported to increase macrophage recruitment via chemokine and inflammatory cytokine release (Xu et al., 2020a).

In vitro *studies*

Mycoplasma gallisepticum

The *M. gal* surface lipoprotein pMGA was reported to interact with chicken apolipoprotein A-I, leading to cilia loss and cell damage *in vitro* (Fuli et al., 2016). Meanwhile, *M. gal*-associated TLR2 activation has been associated with increased ERK pathway-mediated autophagy in macrophages *in vitro* (Lu et al., 2017). Majumder *et al.* later confirmed their findings in an *in vitro* co-culture model of chicken macrophages and TECs, reporting that infection with a virulent *M. gal* strain (R_{low}) induced significantly higher levels of chemotaxis compared to the less virulent R_{high} strain (Majumder and Silbart, 2016).

M. gal-infected TECs were also found to upregulate miRNA-146a, which inhibits TLR-2 signalling and may serve as a counterbalance to the observed pro-inflammatory processes (Majumder et al., 2014). Altered miRNA profiles have been reported in *M. gal*-infected lung cells as well, where they may regulate major signalling processes including the MAPK, Wnt, JAK/STAT and PI3K/Akt/NF-κB pathways (Kang et al., 2019; Y. Zhao et al., 2017a). Several studies have focused specifically on *in vitro* characterisation of individual miRNAs during *M. gal* infection. The miRNA gga-miR-101-3p is upregulated in *M. gal*-infected DF-1 cells, and the subsequent inhibition of host EZH2 (enhancer of zeste homolog 2) expression may impact T cell function (J. Chen et al., 2015). Upregulated gga-miR-24-3p, meanwhile, was reported to decrease proliferation and increase apoptosis by targeting Ras-related protein-B (Yingjie Wang et al., 2021). Conversely, the FOXO3-targeting gga-miR-223 is downregulated during *M. gal* infection, causing a similar phenotype (reduced proliferation, increased apoptosis) *in vitro* (Yin et al., 2021). Finally, Zou *et al.* reported high levels of gga-miR-193a in exosomes derived from *M. gal*-infected primary chicken type II pneumocytes, and internalisation of these exosomes by DF-1 cells decreased proliferation and increased apoptosis and proinflammatory cytokine secretion via the RAS/ERK signalling pathway (Zou et al., 2022). The role of miRNAs in poultry mycoplasma infections is discussed further in Immunology.

Mycoplasma synoviae

As with *M. gal*, much of the proteome of *M. syn* remains uncharacterised, leaving gaps in our knowledge of its pathogenicity and our ability to produce new vaccine candidates (Q. Sun et al., 2022). Bao *et al.* characterised the alpha and beta subunits of the *M. syn* pyruvate dehydrogenase complex E1 (pdhA and pdhB), finding that both were capable of binding chicken plasminogen and were required for optimal binding to the DF-1 chicken cell line *in vitro* (Bao et al., 2021). A similar study was later conducted on the metabolic enzyme dihydrolipoamide dehydrogenase, finding that it also localised to the cell membrane, bound both fibronectin and plasminogen, and was required for binding to DF-1 cells (Qi et al., 2022).

Finally, as mentioned above, a relatively small number of SNPs separate the attenuated *M. syn* vaccine strain MS-H from its parental strain 86079/7NS, and re-isolation of vaccine strains from vaccinated flocks has identified isolates with reversions to the parental sequence specifically in the *obgE*, *oppF* and *gadph* genes (Klose et al., 2022a). A recent *in vitro* study reported that these changes altered the metabolism and growth kinetics of the mutated MS-H *in vitro* (including alterations in peptide/amino acid uptake, glycerophospholipid metabolism, and the arginine deiminase pathway), indicating a potential mechanism underlying the attenuation of this vaccine strain (Klose et al., 2023).

Immunology

Our knowledge of the immune response to *M. gal* and *M. syn* remains rudimentary, but – at least in the case of *M. gal* – progress is being made. The studies reported below illustrate the power of *in vivo* transcriptional studies to generate data that can be used to formulate testable hypotheses for future work and highlight an emerging role for non-coding mRNAs that warrants further investigation. Yet, most studies have been performed in far from field-like conditions, calling for further validation before their conclusions can be fully accepted.

Mycoplasma gallisepticum

M. gal infection causes severe lymphoproliferative lesions along the respiratory tract of both chickens and turkeys; therefore, the immune response to this agent is both friend and foe. What remains unclear is where the protective response ends, and immunopathology begins; this is necessary

knowledge for deepening our understanding of the disease and for the development of improved vaccines.

Immunogenetics

There have not been any notable advances published in this area since 2015.

Innate immunity

In their rare study of the *in vivo* response to *M. gal*, Beaudet *et al.* reported the changing transcriptional profile of the chicken trachea during the first week post-infection: they identified over 2500 significantly differentially expressed genes between *M. gal* R_{low}-infected and control chickens, including many involved in innate immune signalling pathways (TLR, MAPK, JAK/STAT and NOD-like receptor), as well as increased transcription of genes encoding TLRs 4 and 16 and ILs -1β and -8 (Beaudet et al., 2017). A subsequent study of the earliest days post-infection additionally revealed upregulation of expression of genes encoding IL-6, IL-12β and IL-22, among others, in response to R_{low} but not to attenuated *M. gal* strains: a possible hint as to the difference in immunological trajectory leading to protection versus pathology (Beaudet et al., 2019). These data represent an invaluable resource to guide future studies of the early phases of the immune response to *M. gal*, and as a validation benchmark for studies in other, less physiological, systems.

In recent years there has been a growing appreciation of the potential role of miRNAs in the regulation of biological pathways with relevance to diseases of chickens (reviewed in (Wang, 2020)). Following their initial identification of miRNAs that were differentially expressed in the lungs of embryonic chickens infected with *M. gal* (Y. Zhao et al., 2017a), *in vitro* studies in embryonic chicken lung cells and an embryonic chicken fibroblast cell line (DF-1) showed that *M. gal* infection increased expression of miR-142-3p, leading to decreased expression of pro-inflammatory cytokine genes and limiting apoptosis (Yang et al., 2021). Similar experiments by the same group then revealed increased expression of miR-33-5p during infection with *M. gal*, which also inhibited the expression of pro-inflammatory cytokines and of *M. gal*-induced apoptosis-related genes (Y. Sun et al., 2022). Alongside, *M. gal* infection also led to decreased expression of the long-noncoding RNA, lnc90386, which normally binds to, and reduces the effects of, miR-33-5p (Y. Sun et al., 2022). Another miR, miR-130b-3p, has been proposed to assist cells in defending against *M. gal*, with overexpression of this miRNA leading to increased cell proliferation during infection *in vitro* (Yuan et al., 2018); while miR-21, by contrast, promoted proinflammatory cytokine expression by embryonic chicken lung cells and the DF-1 cell line after exposure to *M. gal* (Zhao et al., 2019). Lastly, a recent study showed that miR-181a-5p

contained in exosomes from *M. gal*-infected chicken pneumocytes was taken up by DF-1 cells *in vitro*, where it targeted PPM1B leading to NF-κB upregulation and increased transcription of genes encoding TLR2, MYD88, TNF-α and IL-1β (Sun et al., 2021).

DF-1 cells and chicken embryos have also been used to look at TLR expression and the potential role of these receptors in *M. gal* infection. An initial study of DF-1 cells detected abundant expression of TLR6, but not TLR2, while both TLRs were expressed at comparable mRNA levels in the lungs of embryonic chicks; infection with *M. gal* increased expression of both TLRs in both systems, and led to activation of NF-κB and transcription of inflammatory cytokine genes including those encoding IL-2, IL-6 and TNF-α (Tian et al., 2016). A subsequent study suggested that TLR2 expressed by DF-1 cells has an important role in the response to *M. gal*-derived lipid-associated membrane proteins, again leading to activation of NF-κB and, here, to expression of IL-β (Ying Yu et al., 2018).

The relevance of these *in vitro* findings to *in vivo* infections of chickens remains to be assessed.

Adaptive immunity

A single study has investigated the adaptive immune response to *M. gal* infection in adult chickens. Kulappu Arachchige *et al.* examined the cellular immune response to *M. gal* in the tracheal mucosa of experimentally infected birds with or without prior vaccination: they found that protection was associated with lower levels of transcription of genes encoding IL-2, IL-6, RANTES and CXCL-14, higher levels of IFN-γ, and greater numbers of infiltrating B cells, compared to those seen in unvaccinated birds; while the pathology of primary *M. gal* infection in the absence of vaccination correlated with increased transcription of genes encoding IFN-γ, IL-17, RANTES and CXCL-14, and decreased mRNA expression of IL-2 (Kulappu Arachchige et al., 2021b). NK cells did not appear to play a role in protection (Kulappu Arachchige et al., 2021b).

Complementing this work, Miao *et al.* have been investigating the role of the respiratory microbiota in shaping mucosal immunity in the lung during *M. gal* infection of chickens. They found that the altered microbiota resulting from *M. gal* infection induced inflammatory/oxidative damage when transferred to the trachea of control uninfected chickens; moreover, the authors detected evidence of altered ratios of $CD4^+$-to-$CD8^+$ in chickens receiving *M. gal*-infection microbiota, which was associated with a possible Th1/Th2 imbalance (Miao et al., 2022a). These interesting findings warrant further investigation, including assessment of the possible effects of mycoplasma co-transferred with the tracheal microbiota of infected chickens.

Although most studies of *M. gal* immunology focus on chickens or their derived cell lines, two papers described investigations of the local adaptive immune response to *M. gal* infection in turkeys. Wijesurendra *et al.* observed the infiltration of lymphocytes into the tracheal mucosa of turkeys that were experimentally infected with virulent *M. gal* via the aerosol route two weeks previously (Wijesurendra et al., 2017, 2015); the authors also detected specific antibody responses at seven and 14 days post-challenge, which varied in magnitude according to the *M. gal* strain used but could not be correlated with local B cell infiltration due to the absence of reagents for detecting turkey B cells (Wijesurendra et al., 2017). More detailed analysis of the turkey immune response to infection has yet to be achieved.

Maternal immunity

Although the transfer of maternal antibody to chicks via both the egg yolk and the egg white is known to occur (Hamal et al., 2006), and has the potential to interfere with vaccination – as most recently illustrated for avian influenza (Maas et al., 2011); there have not been any studies of the effect of maternal immunity on either protection from *M. gal* or interference with vaccination against this pathogen. A recent study also reported heritable effects of maternal immune activation upon chicks' ability to mount responses to a model antigen vaccine (Verwoolde et al., 2022), but the impact of these findings on immunity to mycoplasmas affecting poultry is similarly unknown.

Immune evasion

In their study of the adaptive immune response to *M. gal*, Kulappu Arachchige *et al.* also identified signs of immune evasion by the pathogen. When the authors compared the vaccination-induced protective response to that induced by virulent *M. gal* in the absence of prior immunity, they found that *M. gal* induced immune dysregulation in the tracheal mucosa, characterised by high expression of the genes encoding IFN-γ and IL-17, coupled with decreased IL-2 expression, which was associated with fewer $CD8^+$ T cells and B cells infiltrating the area (Kulappu Arachchige et al., 2021b).

Alongside, Zhang *et al.* injected the virulent *M. gal* strain R_{LOW} into the bilateral air sacs of chickens and saw profound changes within the bursa of Fabricus: infected birds exhibited a significant infiltration of inflammatory cells coupled with lymphocyte apoptosis within the first week of infection, which correlated with increased levels of inflammatory cytokines and the expression of genes involved in the production on reactive oxygen species (ROS) in the bursa (Zhang et al., 2020). Ishfaq *et al.* also documented signs of immune evasion/dysregulation during *M. gal* infection, finding that the bursa of

chickens exposed to *M. gal* contained numerous apoptotic cells and expressed high levels of genes encoding proinflammatory cytokines, coupled with evidence of $CD8^+$ T cell depletion (Ishfaq et al., 2021b)

Mycoplasma synoviae

A single study has investigated the immune response to *M. syn*. Omotainse *et al.* reported the infiltration of B cells and $CD4^+$ T cells into the tracheas of chickens inoculated with virulent *M. syn* 7NS intra-ocularly, also finding that infection induced increased mRNA expression of the Th1 cytokine IFN-γ (Omotainse et al., 2022). By contrast, the vaccine strain, MS-H, which is derived from 7NS, elicited a predominantly Th17 response (Omotainse et al., 2022).

Geographic distribution and epidemiology

While many new strategies for the management and control of *M. gal* and *M. syn* are under development (discussed in Control of the disease), our understanding of many basic aspects of their epidemiology (e.g., regional prevalence, transmission patterns, the role of wild birds, etc.) remains limited. Over the past decade, published studies have begun to fill in some of the most pressing research gaps in this field. Many groups have conducted country-level studies of mycoplasma prevalence within poultry farms, using molecular techniques (e.g., PCR targeting the 16S rRNA or *vlhA* gene) or serodiagnostics to detect *M. gal* and *M. syn* and draw conclusions based on small-scale transmission patterns. Meanwhile, the potential roles of wild bird species as reservoirs or maintenance hosts have been explored around the world.

Global situation

The Americas

Most of the recent studies on poultry mycoplasma epidemiology in the USA have focused on the western states (e.g., California, where *M. gal* and *M. syn* have been highly prevalent for decades (Mohammed et al., 1986)). Ramsubeik *et al.* investigated the factors associated with *M. gal* positivity in commercial turkeys in California, diagnosing numerous co-infections with *E. coli* and *M. gal*, *Bordetella avium* or *O. rhinotracheale* (Ramsubeik et al., 2021). An earlier study of small poultry flocks

in this state reported *M. gal* or *M. syn* co-infections in ~37% of birds infected with *A. paragallinarum* (Clothier et al., 2019).

Brochu *et al.* studied the prevalence of bacterial and viral pathogens within non-commercial poultry flocks in Ontario, Canada, reporting that 36% and 23% of animals were positive for *M. syn* and *M. gal*, respectively (Brochu et al., 2019a). Multifactorial respiratory diseases – commonly co-infections with *M. gal*, *M. syn*, *E. coli* and/or *Avibacterium* species – were the most common primary cause of clinical signs or mortality in the tested flocks (Brochu et al., 2019b). A later study in Quebec identified *M. syn* as being particularly widespread in layer farms, though its impact on production parameters was estimated to be relatively small (Bergeron et al., 2021).

M. gal and *M. syn* are also present in the West Indies – in Trinidad, their combined prevalence has been estimated at ~32% within backyard poultry farms (Jordan et al., 2018), and a study of apparently healthy unvaccinated free-roaming backyard chickens on St. Kitts reported seroprevalence of 37% (Bolfa et al., 2019).

In Brazil, currently the world's largest exporter and second-largest producer of chicken meat (Aquino, 2022), poor biosecurity on backyard poultry farms facilitates extremely high prevalence of *M. gal* and *M. syn* (L. G. S. de Oliveira et al., 2017). In 2015, *M. syn* was detected in only 14% of backyard chickens in Minas Gerais state, but its prevalence increased dramatically over the next five years (Batista et al., 2020; Silva et al., 2015, 2021), and it is now one of the primary causes of avian infectious arthritis in the country (Reck et al., 2019). At the commercial level, mycoplasma prevalence has been estimated at ~7% and 35% for *M. gal* and *M. syn* in Brazil's Federal District (dos Santos et al., 2021).

Europe

Several reports from Europe have highlighted the growing prevalence of *M. syn*, which has supplanted *M. gal* as the dominant mycoplasma of poultry in some regions (particularly those with pre-existing control programmes for *M. gal*) (Landman, 2014). *M. syn* is widespread in Spain (Cortés et al., 2021) and is gaining prevalence in Poland (Kursa et al., 2019b), while high levels of *M. syn* have been reported among Portuguese broiler breeder flocks (Moreira et al., 2015). A large-scale study in Belgium reported ~13% prevalence of *M. syn* among broiler chickens, compared to ~3% for *M. gal* (Michiels et al., 2016).

The neighbouring Netherlands instituted a compulsory *M. gal* monitoring and control programme in 2001, which appears to have substantially decreased the prevalence of this pathogen within commercial chicken and turkey flocks (ter Veen et al., 2021). A similar programme was implemented for *M. syn* in 2013 (Landman, 2014).

In the UK, the combined flock-level prevalence of *M. gal* and *M. syn* has been estimated at 45%, but further research and surveillance are needed to improve our understanding of their circulation in these flocks (Ball et al., 2018). Similar findings were reported from Italy; a study in Ragusa confirmed that *M. gal* and *M. syn* are actively circulating in both commercial and rural layer farms, with overall animal-level prevalence of 12.5% for *M. gal* and ~23% for *M. syn* (Galluzzo et al., 2022).

Africa

Relatively few studies of poultry mycoplasma prevalence have been reported from Africa, and most that are available have focused on the continent's more populous countries. Estimates of the prevalence of *M. gal* in Egyptian commercial poultry farms have varied widely (e.g., from ~9% up to 62%) (Abdelaziz et al., 2019; Marouf et al., 2022b). A recent comprehensive investigation of chicken flocks in Giza agreed with the lower end of this range, reporting ~11% seroprevalence, while *M. syn* was slightly more common at 13% (El-Ashram et al., 2021). High levels of mycoplasma infection have also been detected on Algerian poultry farms (though the responsible mycoplasma species were not identified), where they are reportedly controlled via significant overuse of antibiotics (Berghiche et al., 2018).

M. gal is also highly prevalent within the growing poultry production system of Nigeria, with ~74% prevalence reported among apparently healthy layer chickens in the city of Ibadan (Bakre et al., 2021). Meanwhile, *M. syn* was reported for the first time in Ethiopia (Hutton et al., 2017), where *M. gal* also remains widespread in large- and small-scale commercial poultry farms (Habte et al., 2022; Jibril et al., 2018; Shiferaw et al., 2022).

Finally, in South Africa, the prevalence of *M. gal* was estimated at ~56% in rural chicken flocks; alongside other common pathogens like Newcastle disease virus and H6N2 avian influenza, *M. gal* places a heavy economic burden on these small-scale poultry systems and increases the risk of antimicrobial overuse (Simbizi et al., 2021).

Asia, the Middle East and Oceania

M. gal is an established pathogen of farmed poultry across Asia, and the prevalence of *M. syn* has also been increasing. *M. syn* is widespread in China (ShiKai et al., 2017a), though seroprevalence analyses have reported highly variable rates depending on the region studied, with province-level estimates ranging from ~5% to 100% (Xue et al., 2017). A recent study of unvaccinated chicken flocks in the south-eastern Fujian province demonstrated high rates of *M. syn* infection alongside horizontal and vertical transmission (ChenYan et al., 2022). In central China, *M. syn* was reported at a much higher prevalence in layers than in broilers (~74% versus 20%, respectively) (Sui et al., 2022).

Mycoplasmas also impact the rapidly expanding poultry sector in India, where egg and broiler production was rising by ~8-10% annually prior to the COVID-19 pandemic (Mani and Beillard, 2021). Bacterial septicaemia, primarily attributable to *E. coli* with worryingly high levels of antimicrobial resistance, can involve co-infections with *M. gal* and *M. syn* (Kaore et al., 2018; Krishnegowda et al., 2022; Rama Raju et al., 2017), and infections by 3+ respiratory pathogens (e.g., *E. coli*, *M. gal*, *M. syn*, Newcastle disease virus, influenza A virus, etc.) are also common (Chaudhari et al., 2018; Gowthaman et al., 2017). A recent overview of mycoplasma prevalence within Indian commercial breeder and layer farms reported that ~6.5% of tested birds were PCR-positive for *M. gal* and ~24% for *M. syn*, while a further 15.5% were co-infected with both pathogens (Giram et al., 2022). However, the prevalence of *M. gal* in particular may be much higher in broiler breeders, where mycoplasma infections are generally found to be more common (Baksi et al., 2016). Reported *M. gal* prevalence is similarly high in neighbouring Bangladesh (Ganapathy et al., 2021; Raquib et al., 2021; Uddin et al., 2016), where surveillance of poultry mycoplasma pathogens is limited (Chowdhury et al., 2018).

Few recent reports on poultry mycoplasma prevalence have emerged from Southeast Asia. In Vietnam, *M. gal* was the third most common bacterial pathogen of poultry (after *Avibacterium paragallinarum* and *Ornithobacterium rhinotracheale*) in recent studies of the Mekong Delta region (Van et al., 2021, 2020).

Meanwhile, Siddique *et al*. analysed mycoplasma prevalence and antimicrobial resistance among broiler and layer poultry in Pakistan, reporting 61.5% positivity (primarily *M. gal*) and high levels of resistance by both *M. gal* and *M. syn* to the fluoroquinolone enrofloxacin (Siddique et al., 2020). Similar findings were reported from Saudi Arabia, where *M. gal* isolates exhibited increasing resistance to seven of 10 tested antimicrobials (Elbehiry et al., 2016).

In Iran, the reported prevalence of *M. syn* was substantially higher than *M. gal* in commercial poultry flocks of the western Khuzestan region (Gharibi et al., 2018), but *M. gal* remained slightly more widespread in Punjab (Shoaib, 2019). Whether these differences reflect actual regional variables, diagnostic test selection, or both is unclear. Rasoulinezhad *et al.* conducted a larger-scale study of *M. syn* in this country, reporting ~52% and 33% prevalence in backyard and commercial farms, respectively (Rasoulinezhad et al., 2018). *M. gal* and *M. syn* have both also been detected on ostrich farms, which are common in Iran (Moomivand et al., 2017). Finally, phylogenetic analysis of circulating *M. gal* isolates has shown high sequence similarity with Pakistani and Indian strains, pointing to cross-border transmission networks (Rasoulinezhad et al., 2017).

Surveillance, transmission and risk factors

Within infected poultry populations, *M. gal* can transmit to other animals horizontally (via direct contact) or vertically (transovarian, through the egg) (reviewed in (Mugunthan et al., 2023)). Horizontal transmission allows *M. gal* to spread rapidly ($R_0 \geq 4.5$) upon introduction to a flock (Feberwee et al., 2005), with animal-to-animal contact, aerosolised pathogen, contaminated food and fomites, and biofilm formation contributing to its transmission (Chen et al., 2012; Dhondt et al., 2007). Vaccines (primarily the F-strain, ts-11 and 6/85 strains) have been used for decades to limit the spread of *M. gal* and have been partially successful in reducing its associated production losses (Michiels et al., 2016; Whithear, 1996), though new and improved vaccines are needed to improve disease control and reduce antibiotic usage (reviewed in (Ishfaq et al., 2020a) and discussed further in Vaccines).

Like *M. gal*, *M. syn* is capable of both horizontal and vertical transmission, and recent studies of *M. syn* transmission parameters have indicated that vertical transmission was the primary route of infection on Dutch layer farms (Feberwee et al., 2017; ter Veen et al., 2020). The deployment of vaccines against *M. syn* (e.g., the MS-H strain) has been reported to reduce or prevent clinical signs, but it does not appear to prevent infection by field *M. syn* (Dijkman et al., 2017).

Wildlife

The epidemiology of *M. gal* and *M. syn* within wild birds remains poorly understood in many regions, and although available studies generally report low prevalence in these populations, their potential roles in transmission and/or as a pathogen reservoir in certain environments cannot be excluded (Michiels et al., 2016). The emergence of new host tropisms appears to be a relatively rare event –

transmission of *M. gal* from infected American house finches to other avian species is likely a common occurrence, for example, but recent findings suggest that within-host mutations are necessary for the establishment of productive infection in new hosts (i.e., close contact alone is not sufficient) (Staley et al., 2018b).

Many groups have reported their investigations of *M. gal* and/or *M. syn* in specific wild bird populations; for instance, *M. gal* has been detected in pigeons (*Columba livia*) in Iran (Ghohestani et al., 2018), eastern wild turkey (*Meleagris gallopavo silvestris*) in Canada (MacDonald et al., 2019), starlings (*Sturnus vulgaris*) in Iraq (Hamad et al., 2019), and gamebirds in Britain (Welchman et al., 2022). Several studies have been published on the prevalence of *M. gal* among wild bird populations in Germany, but none so far have reported a positive case (Fischer et al., 2022b, 2022a; Liebing et al., 2020; Prüter et al., 2018).

In Brazil, both *M. gal* and *M. syn* have been isolated from wild Muscovy ducks (*Cairina moschata*), feral pigeons, and several species of captive wild bird in the Rio de Janeiro Zoo (Ferreira et al., 2016; Magalhães et al., 2020b, 2020a). Despite this, Brazilian wildlife has not been demonstrated to play a major role in the maintenance or transmission of mycoplasmas, even in areas where large wild bird populations live in close proximity to commercial poultry farms (Guimarães et al., 2016). As important epidemiological variables (e.g., climate, human-animal-wildlife interaction patterns, farm density, etc.) can vary between geographical regions, however, it will be important in the future to expand these studies to cover a wider range of farming systems and wildlife populations.

In the USA, *M. gal* was first detected in house finches in the early 1990s, and it remains a significant bacterial pathogen causing conjunctivitis in this species (Dhondt et al., 2017a; Van et al., 2018). The density of bird feeders, which promote the gathering of many birds in a single location, has been found to be significantly associated with *M. gal* transmission among house finches (Adelman et al., 2015; Moyers et al., 2018; Van et al., 2018). A study of finches in Arizona reported an interesting geographical pattern – *M. gal* was detected in ~12% of birds captured in Green Valley near the border with Mexico, but not in birds from further north, suggesting that the relative lack of connectivity between urban regions and the more remote, arid habitats of southwestern Arizona limits the spread of infectious disease between these bird populations (Staley et al., 2018a).

While house finches are the most commonly observed wild hosts of *M. gal* in the USA, house finch-associated strains of *M. gal* have been detected in several other species belonging to the Fringillidae

and Corvidae families (Allen et al., 2018; Ley et al., 2016). *M. gal* also appears to be common in American raptor species, which have been reported to mount effective immune responses against the pathogen (Wrobel et al., 2016). Finally, a recent increase in the incidence of *M. gal*-associated conjunctivitis among purple finches (*Haemorhous purpureus*) in central New York sparked concern, though purple finch experimental infections and population data indicate that an *M. gal* epidemic would be unlikely to cause significant population decline as it did for house finches (Reinoso-Pérez et al., 2023).

Most studies of mycoplasma prevalence in wild birds have focused on one or a small number of species, making it difficult to draw broader conclusions on the impact of region-specific climatic factors or to extrapolate to other species. To address these issues, Sawicka-Durkalec *et al.* recently conducted a large-scale molecular and phylogenetic investigation of *M. gal* and *M. syn* in >1,100 wild birds from 55 species – however, while ~44% of tested birds were mycoplasma-positive, none were specifically positive for *M. gal* or *M. syn* (Sawicka-Durkalec et al., 2021).

Control of the disease

Current control measures for mycoplasma infections in the poultry industry are based on strict biosecurity practices, particularly in breeder flocks, to maintain the mycoplasma-free status, together with control programmes that include regularly performed diagnostic tests (More et al., 2017), vaccination, and antibiotic treatment using tetracyclines, macrolides (tylosin, tilmicosin), fluoroquinolones (enrofloxacin, difloxacin), and pleuromutilins (tiamulin) (Helmy et al., 2020). In a report commissioned by EFSA, the authors caution that although antibiotic treatments reduce clinical signs of the infection and improve the flock's performance, they do not eliminate *M. gal* infection in a flock and thus vertical transmission of the infection from adult carriers is likely (More et al., 2017). Vaccination to control mycoplasma infections is usually applied in commercial layer flocks, especially with multi-age systems, but there are limitations for their use in other contexts. For example, due to the short life of broilers, vaccination cannot be applied; vaccination is not performed in breeder flocks to avoid positive immunological responses to the vaccine strain during surveillance programmes; and there is not any commercial vaccine available for turkeys (More et al., 2017).

Maintaining effective biosecurity involves regular cleaning and disinfection of facilities where birds are kept, particularly in clearing poultry facilities following *M. gal* outbreaks. As airborne particulate

matter (food, drinking water, feathers, droppings, or dust) can act as a substrate for survival of microorganisms, *M. gal* can survive in in the air of different reservoirs of poultry farms (Adell et al., 2015). The application of an air disinfectant, containing a mixture of Despadac®, monoethylene glycol, and water, using a thermo-nebuliser to reduce airborne *M. gal* in a commercial laying hen house was evaluated in Spain (Adell et al., 2015). Unfortunately, the treatment was not effective in reducing the concentration of mesophilic aerobic bacteria and *M. gal* in this study (Adell et al., 2015). A later study employed a NebuPure™ dispersal unit to produce a semi-dry fog of an electrochemically activated disinfecting solution, and the authors reported that this largely automated system was effective in disinfecting *M. gal*-contaminated facilities under experimental conditions (Evans et al., 2017). These opposing results may be due to key differences in their designs: the facilities tested (isolated steel experimental chambers vs a commercial laying hen house); the nebuliser itself; and the disinfectant aerosolised in each. This method deserves further evaluation, as it may be an effective means to clear facilities and equipment of mycoplasmas and other environmentally deposited avian pathogens.

Policy

M. gal eradication programmes via culling were carried out in Europe during the 1980s when many chicken and turkey flocks were infected with *M. gal*; nowadays, culling is implemented only in *M. gal*-positive breeder farms to avoid disease spread to production farms and other breeder farms (More et al., 2017). The adoption of strong biosecurity measures such as have been applied in the EU for more than 30 years, together with regular diagnostic testing to avoid contamination of poultry breeder flocks by *M. gal* or *M. meleagridis*, are effective in preventing the introduction of disease (More et al., 2017). As neither *M. gal* or *M. meleagridis* are zoonotic or highly contagious agents, infected birds with no clinical signs or lesions can be killed on-farm or in slaughterhouses for human consumption.

The biosecurity risk associated with imports of chilled or frozen chicken meat and meat products for the introduction of exotic *Mycoplasma* species into New Zealand was assessed (Cobb and Smith, 2015) and deemed to be extremely low. The authors estimated the likelihood of exposure via this route to be negligible and concluded that exotic *Mycoplasma* species were not likely to be introduced via this route.

Therapeutics

Antibiotics have been used intensively to treat *M. gal*-infected poultry flocks, which over time has resulted in the emergence and spread of resistant bacteria. A review of antimicrobial resistance in poultry pathogens (Nhung et al., 2017) found that the susceptibility to antibiotics of *M. gal* and *M. syn* field strains from Israel, Jordan, Iran, and Thailand, were (in increasing order): erythromycin, chlortetracycline, enrofloxacin, tylosin, and doxycycline. A more recent systematic review of 23 eligible studies that employed the microdilution MIC test based on the guidelines described by (Hannan, 2000) showed that enrofloxacin, oxytetracycline, and tylosin had the highest number of resistant *M. gal* isolates in most of the geographical distributions (Taiyari et al., 2021). The studies that were included in this analysis were from North and South America, Europe, Asia, the Middle East, and Africa (Taiyari et al., 2021). The most frequent antibiotic to which *M. gal* field isolates showed resistance was enrofloxacin (70% of the countries) followed by tylosin (54% of the countries).

A wide range of MIC values was observed in *M. gal* and *M. syn* isolates from chickens in Pakistan both for oxytetracycline and chlortetracycline (Khatoon et al., 2018); the macrolide tilmicosin was the most efficacious drug with a narrow MIC range. *In vitro* testing has also demonstrated high efficacy by macrolides, with tylvalosin being the most effective drug against both *M. gal* and *M. syn* Egyptian field isolates (El-Hamid et al., 2019). This finding was confirmed in later studies of isolates from broiler and layer chickens in Egyptian farms (Bastamy et al., 2022; Emam et al., 2020) and agrees with other research results for *M. syn* isolates in Central and Eastern Europe (Kreizinger et al., 2017). All *M. syn* isolates collected between 2012 and 2017 in Italy showed high MIC values of erythromycin and enrofloxacin (Catania et al., 2019) but, concerningly, tilmicosin MIC values showed a time-dependent increase, indicating an on-going selection process for resistant strains.

An assessment of antimicrobial susceptibility of *M. gal* and *M. syn* isolates from chickens and turkeys obtained from France, Germany, Great Britain, Hungary, Italy and Spain during 2014-2016 found bimodal MIC distributions for the fluoroquinolone enrofloxacin and the macrolides spiramycin, tilmicosin and tylosin, indicating that both species have sub-populations that are less susceptible *in vitro* to those antimicrobials (de Jong et al., 2021b). Bimodal distributions of MIC values for all drugs tested apart from doxycycline, tiamulin, and florfenicol were found in *M. gal* isolates collected in Italy between 2010 and 2020 (Bottinelli et al., 2022). Interestingly, a statistically significant trend toward low MICs of erythromycin, tylosin, tilmicosin, spiramycin, tiamulin and lincomycin was observed, indicating a return to susceptibility of *M. gal* toward these drugs (Bottinelli et al., 2022). All the studies

undertaken to date emphasise the need to continue studying MIC values to aid veterinarians in selecting appropriate treatments for *M. gal* and *M. syn*.

Studies to understand the molecular bases for resistance development in *M. gal* and *M. syn* have identified mutations in the QRDR of the 23S rRNA, with the A2058G and A2059G mutations the most common (Bekő et al., 2020a, 2019b; Beylefeld et al., 2018; Lysnyansky et al., 2015; Taiyari et al., 2021). The presence of either or both of these point mutations was responsible for macrolide resistance development in *M. gal* and *M. syn* (Ammar et al., 2016b; Bekő et al., 2020a; Lysnyansky et al., 2015), with all tested *M. syn* isolates having a G2057A transition in the 23S rRNA consistent with previously described intrinsic resistance to erythromycin (Lysnyansky et al., 2015). Mutations resulting in amino acid changes in the 50S ribosomal protein L22 were associated with decreased susceptibility to macrolides in *M. syn* (Bekő et al., 2020a). A study of the parC QRDR in *M. syn* strains from China identified a C254T mutation resulting in an amino acid change that gave rise to enrofloxacin resistance (X. Zhang et al., 2022). Comparative proteomic analyses of a tylosin-resistant *M. gal* mutant and a susceptible parent strain identified thirteen proteins as differentially expressed in the resistant strain compared to the susceptible strain (Xi et al., 2015). Among the differentially expressed proteins were enzymes that promote the formylation of initiator tRNA, elongation factors Tu and G, and enzymes involved in energy production. These results, together with previous findings of mutations in 23S rRNA or ribosomal proteins, indicate that resistance to macrolides is achieved through alterations in processes involved in protein translation. First reports were made of a transversion mutation at position 2621 (corresponding to 2611 in *E. coli* numbering) in one of the Egyptian *M. gal* isolates (Ammar et al., 2016b), and a point mutation D420N, which has been associated with quinolone resistance, in a South African *M. syn* strain (Beylefeld et al., 2018).

As previously mentioned, the lifespan of broiler chickens is short (5–6 weeks). There may be times when the treatment of several infections is required, which can result in interactions between antimicrobial drugs (Gbylik-Sikorska et al., 2018). As enrofloxacin and doxycycline were the most frequently detected antibacterial drugs in drinking water samples from dispensers on poultry farms, two studies to determine the impact of trace enrofloxacin on the pharmacokinetics of doxycycline in chickens were carried out (Gbylik-Sikorska et al., 2018, 2016). The highest maximum doxycycline tissue concentration was in the liver of *M. gal*-infected chickens also receiving trace doses of enrofloxacin (Gbylik-Sikorska et al., 2016), and trace amounts of enrofloxacin caused statistically significant changes in the doxycycline pharmacokinetic profile (Gbylik-Sikorska et al., 2018). The authors of these studies recommended that doxycycline should not be administered in combination with enrofloxacin,

and that the water supply systems on farms should be regularly cleaned and tested for antibiotic residues to avoid adverse interactions (Gbylik-Sikorska et al., 2018, 2016).

Alternatives to antibiotics

Given the rise in antimicrobial resistance in *Mycoplasma* species and the need to avoid antibiotic residues in animal products, alternative novel antimicrobials are needed to provide therapeutic choice in controlling infections. Recent high-throughput screening of a library of small molecules identified ten molecules with low MICs (0.78–100 μM) and efficacy against multiple *M. gal* strains (primarily the R-strain) and *M. gal* biofilm (Helmy et al., 2020). Of these ten molecules, six were tested in three-week-old chickens infected with *M. gal*, and two were found to be effective growth inhibitors of *M. gal*. These are promising novel antimicrobials that can be further characterised and developed for therapeutic use (Helmy et al., 2020).

Plant extracts and essential oils may also have antibacterial effects. Tea tree (*Melaleuca alternifolia*) essential oil administered through the feed to naturally *M. syn*-infected laying hens showed positive results compared to different antibiotics (Puvača et al., 2020). A commercial mixture of plant essential oils, Toldin CRD, was tested in broiler chickens experimentally infected with *M. gal* in comparison to tilmicosin (Hashem et al., 2022a). Toldin CRD exhibited immunostimulant and anti-inflammatory activities via significant downregulation of expression of the genes encoding TNF-α and IL-6, reduction of lysozyme, myeloperoxidase, and nitric oxide levels, and increase in IgG levels. Although immunohistochemistry and qPCR results demonstrated statistically significant reductions in the levels of *M. gal* antigens and *M. gal* loads in the Toldin CRD-treated group, these were inferior to tilmicosin (Hashem et al., 2022a).

The potential preventive and therapeutic effects of Chinese herbal medicinal formulae were investigated in chickens infected with the *M. gal* MG-HS strain (Y. Wang et al., 2022). Histopathological analysis showed that these formulae could significantly alleviate the severe respiratory inflammation induced by *M. gal* infection, and that this was associated with reduced expression of *M. gal* adhesion protein pMGA1.2 (Y. Wang et al., 2022). Meanwhile, glycyrrhizic acid inhibited MG-HS strain proliferation *in vitro* and reduced pMGA1.2 expression in the lungs of broiler chickens (Hu et al., 2022). The effect of adding chlorogenic acid extract from Japanese honeysuckle (*Lonicera japonica*) to drinking water was also investigated, with the authors reporting increased body weight and reduced seropositivity after six weeks (Müştak et al., 2015). Understanding the mechanism of action of these

and other plant extracts will allow us to further explore their potential as alternatives to antibiotics for treating mycoplasma infections in poultry.

The protective effects of methylsulfonylmethane, a naturally occurring organosulphur compound, were assessed in *M. gal*-induced inflammatory injury in chicken trachea and HD11 cells (Miao et al., 2022b). The action of methylsulfonylmethane to reduce inflammatory injury was through the repression of NF-κB and the ERK/JNK-MAPK signalling pathway and the suppression of proinflammatory cytokines in both the trachea and HD11 cells (Miao et al., 2022b). The action of baicalin, a flavonoid extracted from *Scutellaria baicalensis* and used as a traditional medicine in East Asia, seems to be via a similar mechanism. Oral baicalin treatment of *M. gal*-infected chicks effectively prevented oxidative stress and splenocyte apoptosis via opposite modulation of NF-κB and Nrf2/HO-1 (Ishfaq et al., 2019). Baicalin treatment also attenuated the level of proinflammatory cytokines and suppressed NF-κB expression at both protein and mRNA levels in the chicken bursa of Fabricius in an *M. gal* infection model (Ishfaq et al., 2021a). Meanwhile, *in vitro* studies have indicated that baicalin treatment may reduce *M. gal*-induced oxidative stress, alleviate lung inflammation, and attenuate the expression of pro-inflammatory genes during infection (Chen et al., 2023; Ishfaq et al., 2021b; JiChang et al., 2019; Wu et al., 2020; Zou et al., 2021).

Vaccines

Mycoplasma gallisepticum

There are various commercially available vaccines for *M. gal*, including those based on inactivated mycoplasmas, a recombinant fowlpox-vectored vaccine, and three live-attenuated vaccines: each has their own advantages and disadvantages (reviewed in (Ishfaq et al., 2020a)). Studies in this area have variously aimed to characterise the existing vaccines further, or to improve upon their performance by the design and testing of novel vaccines.

Current vaccines

Understanding the immune response to live-attenuated vaccine strains can give important insights into protective immunity. Towards this aim, Beaudet *et al.* reported on the early transcriptional profile in the trachea of chickens immunised with the attenuated vaccine strains Mg7 and GT5, finding that, despite marked differences in response to the two strains, low expression of IL-8, IL-1β and CCL4 was a common feature of vaccine exposure that was not seen in birds inoculated with virulent *M. gal* (Beaudet et al., 2019).

Other studies have looked at the route of administration of commercially available vaccines against *M. gal*. While a recent study reported the failure of *in ovo* injection of *M. gal* ts-11 to elicit immune responses in chicks (Alqhtani et al., 2022), more promising data have emerged using the live-attenuated vaccine against *M. gal* F-strain. Elliot *et al.* first trialled the Poulvac Myco F vaccine, produced by Zoetis, and identified a low dose that elicited specific IgM production at six weeks of age in approximately half of *in ovo* vaccinated birds (a comparable response to the normal post-hatch immunisation method, but much earlier), at the cost of approximately 10% mortality in injected eggs (Elliott et al., 2018), and was unaffected by the post-immunisation egg disinfection step that is used during commercial *in ovo* injection protocols (Elliott et al., 2020). Moreover, those birds that had responded to the *in ovo* vaccine were able to transmit the vaccine strain to their pen-mates, further increasing vaccine responses across the flock (Elliott et al., 2019). The same group most recently investigated how early the IgM response began to emerge after hatching, finding that detectable levels of antibody were present as early as seven days post-hatch (Elliott et al., 2022). *In ovo* administration of the 6/85 vaccine at a medium dose ($1.73x10^2$ CFU) was also lately shown to have a significant protective effect on hatchlings while avoiding the increased mortality associated with higher doses (Alqhtani et al., 2023). Formal establishment of the onset and extent of protection following this method of immunisation has yet to be published, but the comprehensive results of these initial and logically developed studies are highly promising.

In the field, infection of chickens with common avian pathogens at the time of *M. gal* vaccination may occur; therefore, it is important to understand the impact that this might have on vaccine efficacy. Recent studies found that responses to live-attenuated *M. gal* vaccines were adversely impacted by co-infection with chicken anaemia virus (Kulappu Arachchige et al., 2021a; Prezotto et al., 2016) and infectious bursal disease virus (Kulappu Arachchige et al., 2021a), while they were not significantly affected by co-infection with Newcastle disease virus (Riaz et al., 2021).

Novel vaccines

Inactivated vaccines

Marouf *et al.* trialled a locally produced formalin-inactivated pentavalent vaccine against a range of pathogens affecting chickens: *M. gal*, *M. syn*, and *Salmonella enterica* serovars Typhimurium, Enteritidis, and Kentucky (Marouf et al., 2022a). The authors found that immunising chicks at one and four weeks of age reduced mortality from either *M. gal* or *M. syn* infection from 100% at three weeks, to zero; moreover, vaccinated chicks appeared to clear the bacteria during the first week post-

infection, while control animals continued to shed the pathogens up to two weeks post-infection (Marouf et al., 2022a). Protection was associated with high levels of *M. gal*- and *M. syn*-specific antibody production, which was rapidly elicited by the initial vaccination and boosted by the second (Marouf et al., 2022a). These data show the potential of such a vaccine to protect against a suite of bacterial diseases affecting chickens and warrants further investigation in larger study groups, and in different species.

The means of inactivation used during the preparation of vaccines from live microorganisms can affect the availability and immunogenicity of some antigens. Atalla *et al.* reported the application of 1, 5-iodonapthylazide followed by UV light exposure to *M. gal*, finding that this protocol enabled the retention of lipoproteins and was able to elicit significant titres of *M. gal*-specific antibodies at two weeks post-immunisation of chickens (Atalla et al., 2015).

Taking a different approach, Kumosani *et al.* used solubilised total antigens of *M. gal* and H9A2 avian influenza virus, encapsulated in cationic liposomes with or without immune-stimulating *Echinacea* extract, to twice immunise groups of 20-day-old broiler chicks by aerosol exposure, before virulent challenge with both pathogens one week after boosting (Kumosani et al., 2020). This large and well-conducted study found that that highest concentration of specific antibodies was elicited in the presence of echinacea extract, and that the vaccine conferred significant protection from *M. gal* colonisation as well as from losses in productivity associated with infection (Kumosani et al., 2020). However, in the absence of commercially available vaccine groups for comparison, it is difficult to know whether this approach represents an improvement on the currently available resources for disease control.

Live-attenuated vaccines

Following their early work showing that the commercial live-attenuated ts-11-based vaccine "Vaxsafe MG" contained two *M. gal* variants, one of which retained a functional GapA cytadhesin gene – designated ts-304 – and the other not (Kanci et al., 2004), researchers at the University of Melbourne have continued to test and characterise the $GapA^{+}$ variant as a vaccine candidate. First demonstrating that the strain was safe and effective at a range of doses in chickens (Shil et al., 2011), the researchers went on to show that a single immunisation of ts-304 could protect chickens from virulent challenge four weeks later (Kanci Condello et al., 2020b), with protection lasting to the final timepoint of 57 weeks post-immunisation, representing life-long immunity for commercial layer or breeder chickens (Kanci Condello et al., 2020a).

Tseng *et al.* also reported the development of a novel rationally attenuated *M. gal* strain and tested its safety and efficacy in chickens (Tseng et al., 2017b). Following their identification of the *M. gal oppD* gene as encoding a putative oligopeptide transporter acting as a virulence factor (Tseng et al., 2017a), the authors generated an attenuated Ap3AS lacking this gene, and used it to immunise five-week old chickens once before challenging them with the virulent parental strain two weeks later (Tseng et al., 2017b). The attenuated strain did not induce antibodies against *M. gal*, but it did elicit an antibody response that was comparable to those reported for the commercial ts-11 vaccine by four weeks after immunisation; moreover, the *OppD* mutant protected chickens from pathology when exposed to virulent *M. gal* (Tseng et al., 2017b).

Furthermore, despite early studies that revealed a lack of efficacy (Alessandri et al., 2005) or even pathology (Lin and Kleven, 1982; My and Sh, 1984) induced by immunisation of turkeys with live-attenuated vaccines against *M. gal* that elicited a degree of protection in chickens, evidence is emerging that ts-304 is also effective in this species. Wijesurendra *et al.* tested the $GapA^{+}$ ts-11 vaccine strain in turkeys, finding evidence of reduced T cell infiltration into the trachea which was correlated with less severe disease after challenge with the virulent Ap3AS strain; moreover, despite an absence of detectable antibody induction after vaccination, immunised turkeys did raise significantly higher antibody responses to challenge than their non-immunised counterparts (Wijesurendra et al., 2017). More detailed analysis of safety and efficacy at a range of doses led the group to conclude that *M. gal* ts-304 was a safe and effective vaccine for turkeys, able to elicit long-lasting protection in the absence of adverse effects (Kanci et al., 2018).

Commercially available live-attenuated vaccines are based on the *M. gal* 6/85, F, or ts-11 strains, which vary in their virulence, immunogenicity, and protective efficacy; however, few data exist on the potential of the K-strain of *M. gal* as a vaccine candidate. Ferguson-Noel *et al.* initially reported that the K2101 strain of *M. gal* was both a safe and an effective vaccine in three-week-old chickens (Ferguson-Noel et al., 2012), then went on to show that the K-strain vaccine performed as well as the F-strain vaccine in both broiler and layer chickens (Ferguson-Noel and Williams, 2015).

Subunit vaccines

A single study reported promising results from a novel plant-produced vaccine against *M. gal.* Shi *et al.* generated genetically engineered wheat expressing the T1 gene to produce seeds containing this protein, as an oral vaccine for chickens; after two feedings of powdered seeds two weeks apart, chicks

mounted specific antibody responses and were similarly protected from *M. gal* challenge compared to birds receiving a commercial eye-drop administered live-attenuated vaccine (Shi et al., 2023). The clear advantages offered by plant-based vaccine production, alongside the ease of administration of oral vaccines, justify further follow-up of this interesting work.

Vectored vaccines

Zhang *et al.* reported comparable protection to a commercially available F-36 live-attenuated *M. gal* strain by twice immunising week-old chicks with a recombinant adenovirus bearing *M. gal* TM-1 protein, and challenging them with virulent *M. gal* two weeks later; interestingly, the level of protection from *M. gal* was the same when the adenoviral vector also expressed the S1 spike glycoprotein of infectious bronchitis virus, marking this approach as promising for both mono- and bi-valent vaccination of chickens (Zhang et al., 2018). It would also be interesting to trial this vaccine in turkeys.

Adjuvants

There have not been any notable advances published in this area since 2015.

Mycoplasma synoviae

Current vaccines

Omotainse *et al.* reported that the tracheal response of chickens to the MS-H vaccine strain was characterised by the transcription of IL-17A and the infiltration of $CD4^{+}CD25^{-}$ T cells, B cells and macrophages (Omotainse et al., 2022).

Novel vaccines

A single report has been published on a novel inactivated vaccine targeting *M. syn.* Gong *et al.* subcutaneously immunised day-old broiler chicks once with BEI-inactivated *M. syn* strain CHN-WF224-2016 formulated with different adjuvants and challenged them six weeks later with the virulent *M. syn* strain CHN-BXJ2-2015 (Gong et al., 2020). The authors found that, compared to chitosan- and sodium polyacrylate- based adjuvants, the mineral oil-based adjuvant elicited superior cellular and antibody responses that were associated with high levels of protection (Gong et al., 2020).

Conclusions

M. gal and *M. syn* remain highly prevalent and economically damaging pathogens of poultry across much of the world. Key to the future containment and control of these mycoplasmas will be the expansion of existing surveillance networks, which will likely require international coordination between policymakers and researchers to accurately map transmission patterns, animal movement networks, and risk factors for *M. gal* and *M. syn*. Improving our capacity to track the spread of different poultry mycoplasma strains will also allow the targeted use of disease treatment and control measures, benefitting efforts to reduce the massive overuse of antimicrobials to which *M. gal* and *M. syn* are prime contributors.

In the meantime, our understanding of the fundamental biology, infection pathways, and host-pathogen interactions of these mycoplasmas has been greatly expanded over the past decade, though many of their gene products remain uncharacterised. The application of emerging technologies (e.g., the CRISPR/Cas system, which was recently adapted to *M. gal* (Ipoutcha et al., 2022)) promises to expand the existing repertoire of laboratory techniques for investigating the molecular biology, pathogenesis and immunity of *M. gal* and *M. syn*. The many promising new diagnostic tests and vaccine candidates described above will also need to be tested under a variety of field settings to ensure that they are broadly applicable for *M. gal* detection and control.

Future research priorities

Based on the available literature and expert opinion, we suggest that the following areas of research into mycoplasmas affecting poultry should be considered high priority:

Biology of the pathogens

- *Continuing generation of complete genome sequences to support molecular and epidemiology studies*
- *High-throughput characterisation of* M. gal *and* M. syn *proteomes and smaller-scale studies of individual proteins of unknown function*

Diagnosis

- *Field validation of isothermal molecular diagnostic assays to facilitate pen-side use*

- *Replacement of agglutination tests with validated ELISAs*
- *Standardisation and validation of in-house serodiagnostics against commercial standards*

Pathogenesis

- *Continued identification and functional characterisation of putative virulence factors*
- *Further assessment of the poultry microbiome, its variations between breeds and species, and its interactions with* M. gal *and* M. syn

Immunology

- *Immunogenetics of resistance/susceptibility to mycoplasma-driven disease in commercial poultry species*
- *Studies to discriminate between a protective versus pathologic immune response, and the factors driving the emergence of one over the other*
- *Understanding of the influence of maternal immunity on resistance/susceptibility to disease caused by mycoplasmas and how this should inform optimal timing of vaccination of chicks/eggs*

Epidemiology

- *Expanded surveillance of* M. syn *in domestic poultry flocks, particularly in Africa*
- *Standardisation of prevalence studies (e.g., sample types, diagnostic assays, etc.)*
- *Clarification of the importance of vertical transmission in the spread and maintenance of* M. syn
- *Large-scale (e.g., multi-species) investigations of* M. gal *and* M. syn *in wild bird populations*
- *Characterisation of transmission dynamics at interfaces between domestic and wild birds*
- *Impact of ecological/climatological variables on* M. gal/M. syn *maintenance and transmission*

Control

- *Assessment of antimicrobial susceptibility in veterinary Mycoplasma spp. requires standardised laboratory methods and agreed interpretive criteria for clinical breakpoints to facilitate correlation of MICs with in vivo efficacy of antibiotics*
- *Development of novel effective means of controlling* M. gal *in light of efforts to reduce reliance on antibiotics*
- *Further study of the potential of identified lead candidates for plant-derived antimicrobials suitable for use against* Mycoplasma *species affecting poultry*

Conclusions

The studies described in this report present important advances in all fields of veterinary mycoplasma research, from basic biology to vaccine development and policy recommendations. Great strides have been made in characterising mycoplasma proteins, host-pathogen interactions (including transcriptional and metabolic changes), and the mechanisms of immune evasion. However, many mycoplasma gene products remain uncharacterised, and post-translational processing events that may control the functions of virulence factors (e.g., adhesion proteins) are not well-understood. The great diversity of veterinary mycoplasmas means that approaches for studying one species are not always suitable for studying another, and the development of molecular biological tools targeted to individual species (e.g., new sequencing and genome editing protocols, *in vitro* models, etc.) will therefore continue to be an important research avenue. The continuing generation and annotation of complete sequences (and the periodic re-annotation of past genomic studies in light of new data) is also an important priority.

Meanwhile, the effective application of next-generation techniques (e.g., single-cell RNA-seq) to veterinary immunology relies on the development of a broader selection of immunological reagents and funding for basic studies of host species immunology. Experimental challenge models often focus on replicating gross pathological findings but exclude the effects of transmission routes and field-like levels of pathogen exposure. While it is commonly noted that route of vaccine administration will modify immunological outcomes, for example, the extent of mucosal immunity, the field appears slow to act on the impact of the challenge method and the associated potential for creating research artifacts. While replicating near field-like challenges may be technically difficult, such a system would allow a higher level of confidence in the data generated, ultimately requiring fewer studies/animals to definitively resolve a research question compared to multiple (sometimes contradictory) reports stemming from non-field-like artificial challenge models.

The same is often true for cell lines and *in vitro* cultured mycoplasmas, which may not faithfully replicate *in vivo* processes and host-pathogen interactions – therefore, it is important that key findings be proven replicable in primary cells or *in vivo*. Otherwise, we run the risk of basing vaccination strategies and other control measures on non-physiological data, causing problems or failures further down the line.

The properties of many pathogenic veterinary mycoplasmas – including high transmissibility, vertical transmission, and frequency of chronic infections – make surveillance a complicated endeavour,

especially in rural and/or remote areas with limited access to veterinary services and diagnostic laboratories. While mycoplasma prevalence reports have become more frequent in these areas, there is a pressing need to tie these small-scale studies together to draw a cohesive picture of disease transmission patterns and risk factors. This standardisation should go hand-in-hand with the development and validation of effective, reproducible, and (ideally) field-deployable diagnostics to reduce uncertainty and allow direct comparison between among datasets.

Many gaps remain in the linking of research findings with practical applications and policy as well. Socio-political considerations impact the allocation of disease surveillance and control resources, and therefore affect the transmission patterns of clinically significant veterinary mycoplasmas. Some mycoplasma-associated diseases are relatively neglected due to their primary endemic geographical locations (e.g., CBPP in sub-Saharan Africa and parts of Asia) or the species at risk, despite the huge impact these diseases have on the livelihood of affected farmers. In the case of CBPP, several successful partnerships between field centres and high-resource settings have enabled some access to additional technology for detailed analysis, and this should be encouraged to accelerate disease research and improve the efficacy and availability of current vaccines.

Altogether, our understanding of veterinary mycoplasmas and our repertoire of disease surveillance and management methods have grown significantly over the past decade. The studies covered in this report encourage optimism in the future of veterinary mycoplasma control while highlighting the particular importance of increased standardisation and coordination between laboratories and nations in combating these diseases. The authors of this report hope that it will be a useful resource for researchers and policymakers as we enter the next decade of research into these complex organisms.

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Appendices

Abbreviations

AHAW	Animal Health and Welfare
AMPV	Avian Metapneumovirus
ARS	Agricultural Research Service
BEI	Binary Ethyleneimine
BMDC	Bone Marrow-derived Dendritic Cell
bp	base pair
BRD	Bovine Respiratory Disease
CBPP	Contagious Bovine Pleuropneumonia
cELISA	competitive ELISA
cgMLST	core genome Multilocus Sequence Typing
C. M. haemobos	*Candidatus* Mycoplasma haemobos
C. M. haemosuis	*Candidatus* Mycoplasma haemosuis
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DC	Dendritic Cell
DEG	Differentially Expressed Gene
DIVA	Differentiating Infected from Vaccinated Animals
DNA	Deoxyribonucleic Acid
dpi	days post-infection/immunisation
EBL	Embryonic Bovine Lung
ECM	Extracellular Matrix
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FISH	Fluorescence *In Situ* Hybridisation
GBP	Great British Pounds
GlpO	l-α-glycerol-3-phosphate oxidase
HRM	High-Resolution Melting
IAV	Influenza A Virus
IFN	Interferon

Ig	Immunoglobulin
IGS	Intergenic Spacer
IHC	Immunohistochemistry
IL	Interleukin
IM	Intramuscular
JAK	Janus Kinase
kb	kilobase(pair)
LAMP	Loop-Mediated Isothermal Amplification
Lpp	Lipoprotein
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
MAMA	Mismatch Amplification Mutation Assay
MAPK	Mitogen-Activated Protein Kinase
MBL	Mannose-Binding Lectin
MEC	Mammary Epithelial Cells
Mfl	*Mycoplasma flocculare*
M. gal	*Mycoplasma gallisepticum*
MHC	Major Histocompatibility Complex
Mhp	*Mycoplasma hyopneumoniae*
Mhr	*Mycoplasma hyorhinis*
Mhs	*Mycoplasma hyosynoviae*
MIC	Minimum Inhibitory Concentration
miRNA	microRNA
MLST	Multilocus Sequence Typing
MLVA	Multilocus Variable number tandem repeat Analysis
Mmm	*Mycoplasma mycoides* subspecies *mycoides*
MOI	Multiplicity Of Infection
MPS	Mycoplasmal Pneumonia of Swine
M. syn	*Mycoplasma synoviae*
MYD88	Myeloid Differentiation Factor 88
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor-κB
NK	Natural Killer
NOD	Nucleotide-binding Oligomerization Domain-containing protein

OIE	Office International des Epizooties (now the World Organisation for Animal Health)
PAM	Porcine Alveolar Macrophage
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PCV	Porcine Circovirus
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
qPCR	quantitative real-time Polymerase Chain Reaction
QRDR	Quinolone Resistance-Determining Region
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
ROS	Reactive Oxygen Species
RPA	Recombinase Polymerase Amplification
sIAV	swine Influenza A Virus
sIgA	secretory Immunoglobulin A
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
SPLUNC1	Short palate, lung, and nasal epithelium clone 1
ST	Sequence Type
STAR-IDAZ	Strategic Alliance for Research into Infectious Diseases of Animals and Zoonoses
STAT	Signal Transducer and Activator of Transcription
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TOC	Tracheal Organ Culture
UK	United Kingdom
USA	United States of America
USD	United States Dollars
USDA	United States Department of Agriculture
VNTR	Variable Number Tandem Repeat
WOAH	World Organisation for Animal Health

Details of searches

The literature search terms provided below were used to interrogate the CAB Abstracts database (www.cabdirect.org) on 31st January 2023. The searches were repeated using the PubMed database (which has a shorter lag time between publication and updating) on 8th May 2023, to capture any new literature published during the report-writing period.

Topic	Search terms	Date range
Mycoplasma mycoides subsp. *mycoides*	CBPP or "Mycoplasma mycoides subsp. mycoides" or "bovine pleuropneumoni*" or (pleuropneumonia and cattle) or "contagious bovine pleuropneumonia"	1/1/2012 – 31/1/23
Mycoplasma bovis	"mycoplasma bovis" and (cattle or bison or buffalo* or bt:bovidae)	1/1/2012 – 31/1/23
Other mycoplasmas affecting cattle	"Mycoplasma alkalescens" OR "Mycoplasma bovigenitalium" OR "Mycoplasma bovirhinis" OR "Mycoplasma bovoculi" OR "Mycoplasma californicum" OR "Mycoplasma canadense" OR "Mycoplasma dispar"	1/1/2015 – 31/1/23
Mycoplasmas affecting swine	("Mycoplasma hyopneumoniae" OR "Mycoplasma hyorhinis" OR "Mycoplasma hyosynoviae") or (mycoplasm* and pigs)	1/1/2015 – 31/1/23
Mycoplasmas affecting poultry	("Mycoplasma gallisepticum" OR "Mycoplasma gallopavonis" OR "Mycoplasma meleagridis" OR "Mycoplasma synoviae" OR "Mycoplasma anatis" OR "Mycoplasma iowae") or (mycoplasm* and (fowls or chickens or turkeys or ducks or geese or poultry or quails))	1/1/2015 – 31/1/23

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Conflict of Interest Statement

The authors declare no conflict of interest.

Additional Resources

Please see the following websites for additional information and/or resources around the content and aims of this report:

STAR-IDAZ

USDA ARS

PubMLST database

Vet immunology toolkit (UK)

http://www.vetimm.org/ (USA)