Advances in *Mycoplasma hyopneumoniae* diagnostics and implications for disease control

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Dedication

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Abstract

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is one of the most significant pathogens in swine, causing a respiratory disease that affects animal welfare and decreases animal production. Disease diagnostics are the cornerstone for the implementation of measures aiming at the detection and control of pathogens, like *M. hyopneumoniae*. However, some aspects of disease diagnostics, such as PCR result interpretation or bacterial viability determination, are challenging and subject to improvement. The general objective of this thesis was to contribute to the advancement of *M. hyopneumoniae* diagnostic capabilities, to be applied in disease control.

Unforeseen laboratory testing results, such as the detection of *M. hyopneumoniae* by PCR in processing fluids (PF), can occur and require further analysis. The detection dynamics and the possible origin of the genetic material present in PF were evaluated in different farm conditions. Detection of the bacterium was a consistent finding in positive farms, and the origin of the genetic material could be traced down to cross-contamination, highlighting the importance that the persistence of nucleic acids in the environment can have for proper PCR result interpretation.

Although the extensive resistance to degradation of DNA in the environment has been established in certain fields of knowledge, there was limited information on the detection dynamics of DNA from non-viable *M. hyopneumoniae* in various conditions. Thus, the detection of inactivated *M. hyopneumoniae* was explored both *in vitro*, using cultured bacteria in the closed environment of the culture tubes, and *in vivo*, inoculating pigs with non-viable cells. Combined results indicated that, while DNA detection of non-viable bacterial cells persisted *in vitro*, this did not occur *in vivo*, as DNA was not detectable by PCR in the organism of the pigs, not even soon after inoculation. Based on the obtained results, the development of a culture-independent method to assess the viability of *M. hyopneumoniae* in clinical samples was considered essential.

A viability, RNA-based PCR assay, targeting *M. hyopneumoniae* messenger RNA (mRNA) was developed and compared to DNA-based PCR in regard to their ability to differentiate viable from inert bacteria. The developed RNA-based PCR assay detected only viable or very recently inactivated *M. hyopneumoniae*, while the DNA-based PCR consistently detected cells irrespective of their viability status. Changes in *in vitro* growth activity over time were only observable via RNA-based PCR.

Furthermore, the applicability of the viability PCR assay to evaluate *M. hyopneumoniae* viability post-antibiotic treatment in infected pigs was investigated. Results showed differences between the detection of bacterial DNA and mRNA, suggesting that DNA-based PCR assays may overrepresent the population of viable bacteria. Thus, the use of diagnostic methods that allow the relative quantification of viable cells may be more suitable to determine the efficacy of antibiotic treatments than DNA-based methods. However, the clinical or epidemiological implications of such differences, especially in the chronic phase of infection, are unknown, and further research is warranted.

Overall, the new information gathered in this thesis indicate that improved diagnostics are critical for the control of the disease caused by *M. hyopneumoniae*. The data presented here will impact the work of swine practitioners and producers, and will contribute to a more sustainable pork production.

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General Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary etiologic agent of enzootic pneumonia, a prevalent disease in the porcine industry worldwide, which impairs animal health, welfare, and productivity (Pieters & Maes, 2019). The disease is clinically characterized by non-productive cough of variable severity and duration, high morbidity, low mortality, and is mainly expressed in grow-finishing pigs. *Mycoplasma hyopneumoniae* is also a primary pathogen of the Porcine Respiratory Disease Complex, a multifactorial respiratory condition for which this bacterium is considered to facilitate the participation of other viral and bacterial agents (Saade et al., 2020). Economic expenses associated with disease occurrence include increased antibiotic usage and vaccine application, reduction in growth rates, increased feed conversion, mortality and culling rates. Production losses have been estimated to be approximately $7.00 per pig marketed (Silva et al., 2019), and even higher when the disease occurs in combination with other pathogens (Dykhuis-Haden et al., 2012). Also importantly, *M. hyopneumoniae* infections are associated with animal welfare issues and decreased morale of farm personnel.

The implementation of control measures is widely used in the field to reduce the detrimental effects of the infection. Additionally, strategies to eliminate the infection from swine herds are on the rise, and are observed as the means for absolute disease control (Yeske et al., 2020). However, all the interventions aiming at monitoring, controlling or eliminating *M. hyopneumoniae* rely on methods to accurately detect the bacterium and diagnose the disease. Therefore, the objective of this thesis is to provide new information that can contribute to improving the diagnostic capabilities available for

M. hyopneumoniae. In particular, new insights on the nature and characteristics of molecular detection and the viability status for the bacterium in various scenarios are presented.

Several diagnostic challenges exist for *M. hyopneumoniae*, and are basically associated with the intrinsic characteristics of the bacterium and of the disease it produces in pigs. First, the course of the infection with *M. hyopneumoniae*, as with other respiratory infections caused by *Mycoplasma* species, is chronic by definition (Dobbs et al., 2009). Thus, it is evident that the efficiency of the immune system is imperfect at clearing the pathogen from the respiratory system. This might be associated with different mechanisms of immune evasion elicited by *M. hyopneumoniae* cells. In fact, *M. hyopneumoniae* can persist in the lungs for long periods (Fano et al., 2005; McDowell et al., 2023; Pieters et al., 2009), and infected pigs can remain contagious to others for at least 200 days, which is long after disease recovery (Pieters et al., 2009). Therefore, the bulk of the infection period in general, and of the chronic phase in particular, is characterized by an asymptomatic, carrier state, as pigs may remain chronically infected for several months. Second, *M. hyopneumoniae* is a very fastidious bacterium to grow in culture, with isolation rates as low as 2.6% (Friis, 1971), mostly due to the highly specific nutritional requirements and the optimal incubation conditions needed (Friis, 1975; Kobisch & Friis, 1996). Therefore, bacterial isolation is not routinely performed for diagnostic purposes, and the detection of *M. hyopneumoniae* in clinical samples is mainly accomplished through molecular techniques. However, the current detection methods, based on DNA amplification via PCR, are unable to provide information about the viability of the detected bacteria. Taken together, current diagnostic challenges have important implications, not only in diagnostic instances, but also from the point of view of better understanding the pathogenesis and the epidemiology of the disease. Determining the viability of *M. hyopneumoniae* is essential to, for example, defining the biological relevance of an unexpected detection of the bacterium, to assess the efficacy of antibiotic treatments, or to characterize the clearance of infection at late stages of the chronic phase of infection, among others. To date, information regarding the persistence of detection of genetic material from non-viable *M. hyopneumoniae* in various conditions is limited. Similarly, the development of culture-independent techniques to determine the viability of *M. hyopneumoniae* has been poorly explored.

In chapter 2, PCR detection of *M. hyopneumoniae* in processing fluids was studied, and its association to various factors was assessed. The extrapulmonary detection of the bacterium was a significant finding, since *M. hyopneumoniae'*s habitat is considered to be restricted to the respiratory tract. Additionally, the possible origin of the detected genetic material was hypothesized. The results obtained in this study stressed the necessity to investigate the persistence of detection of *M. hyopneumoniae* DNA in different conditions.

Understanding the nature and the dynamics of detection of the targeted genetic material in PCR testing is a prerequisite for the correct interpretation of results. However, different aspects can affect DNA detection, such as the viability of the targeted microorganism, the environmental conditions in which DNA is located, the presence of inhibitors and the immune system of the host, among others. Thus, in chapters 3 and 4, the DNA detection of non-viable *M. hyopneumoniae* was investigated *in vivo* and *in vitro*, respectively.

As mentioned above, the determination of *M. hyopneumoniae* viability in particular circumstances has broad implications in various diagnostic scenarios, such as when evaluating the efficacy of antibiotic treatments aimed at reducing bacterial load, or when investigating the shedding of the bacterium at late stages of chronic infections. However, the benefits of determining bacterial viability are not restricted to just diagnostic purposes, as it can also help gain knowledge about different aspects of the epidemiology and the pathogenesis of *M. hyopneumoniae*, such as in identifying alternative transmission routes or characterizing the features of extrapulmonary *M. hyopneumoniae* cells. Therefore, in chapter 4, a culture-independent method of determining *M. hyopneumoniae* viability, based on the molecular detection of messenger RNA by reverse-transcriptase PCR, was developed and compared to DNA detection by real-time PCR.

Antibiotic treatments are one of the main measures implemented to control and reduce the negative effects of *M. hyopneumoniae* infection. Indeed, antibiotic treatment is required when the clinical disease exists, to prevent economic losses due to decreased performance and to protect animal welfare. Additionally, antimicrobials are frequently employed in *M. hyopneumoniae* elimination programs (Yeske et al., 2020). As for any other disease control interventions, it is essential to assess the effectiveness of a particular antibiotic treatment on the clearance of the pathogen from the host. The positive effects of treatment can be measured using different metrics, such as clinical or pathological scoring systems (Garcia-Morante et al., 2016; Nathues et al., 2010). Additionally, *M. hyopneumoniae* DNA detection by PCR is commonly used to assess the efficacy of antibiotic treatments. However, the interpretation of PCR detection results can be especially challenging in this context, as persistence of bacterial detection post-treatment has been described for different types of antibiotics, and bacterial viability cannot be inferred from PCR assays based on bacterial DNA detection. Thus, in chapter 5, the developed, RNA-based PCR assay was used to evaluate the viability of *M. hyopneumoniae* post-antibiotic treatment in experimentally infected pigs under commercial conditions.

The knowledge obtained from this thesis will help improve the diagnostic capacities for *M. hyopneumoniae*, which are the bases for successful surveillance, control and elimination strategies. Additionally, this thesis provides the initial steps towards further research that can be applied to gain knowledge in different areas of the biology of *M. hyopneumoniae*. The presented information will impact the lives of swine veterinarians, producers and diagnosticians, and will contribute to a more responsible, sustainable and efficient animal protein production.

Chapter 1: Literature Review

1.1. Biology of the genus *Mycoplasma*

The genus *Mycoplasma*, belonging to the class Mollicutes, was described for the first time in 1898, when Nocard and Roux isolated an agent assumed to be the cause of bovine pleuropneumonia and named it *microbe de la peripneumonie*, which was later classified as *Mycoplasma mycoides* (Razin & Hayflick, 2010). Mycoplasmas are distinguished from other eubacteria by their lack of cell wall, minute genome size (580-1,380 kb), low C-G genome content (23-40%), cholesterol dependence for growth, and UGA codon usage, which encodes for tryptophan in Mycoplasmas and is a stop codon in other organisms (Razin, 1992). Mycoplasmas, considered the smallest self-replicating prokaryotes known, with a diameter as small as 0.2 μm, gave rise to the investigation of the 'minimal cell' concept, defined as the maximum level of biologic simplicity for an organism to grow and replicate (Morowitz & Tourtellotte, 1962). The extreme simplicity of mycoplasma cells was key to the understanding of this concept, as cells essentially contain the minimum number of organelles for growth and reproduction: a very small circular double-stranded DNA molecule providing the genetic information, reduced numbers of ribosomes for protein assembly, and a plasma membrane separating the cytoplasm from the external environment.

Phylogenetically, Mollicutes are considered to have evolved from walled, Gram-positive eubacteria, and have been described literally as "merely degenerate clostridia" (Woese, 1987). It is hypothesized that during the rapid, reductive evolution from the Gram-positive bacillus-lactobacillus-streptococcal lineage, Mycoplasmas lost the cell wall and many biosynthetic pathways, becoming dependent of a host for the obtention of essential components for maintenance and reproduction, such as amino acids, fatty acids and cholesterol, among others (Sirand-Pugnet et al., 2007). Indeed, the parasitic mode of life is critical for *Mycoplasma* survivability, and all known Mycoplasmas are parasites of humans, vertebrates, plants and arthropods (Citti & Blanchard, 2013).

The taxonomic classification of the genera within the Mollicutes class – *Mycoplasma, Acholeplasma, Anaeroplasma, Spiroplasma* and *Ureaplasma* -has been in constant evolution since the implementation of sequence data of the 16S rRNA gene as a phylogenetic tool, accompanied by phenotypic and serologic characterization (Maniloff, 2002). In recent years, nomenclature changes within the members of the Mycoplasmatales were proposed by Gupta et al. (2018), specifically involving the introduction of the names *Mesomycoplasma* gen. nov., (including *Mesomycoplasma hyopneumoniae*, *Mesomycoplasma hyorhinis* and *Mesomycoplasma flocculare)*, *Metamycoplasma* gen. nov., (including *Metamycoplasma hyosynoviae)*, and *Mycoplasmoidaceae* fam. nov., in which the hemotropic *Mycoplasmas*, including *Eperythrozoon suis*, were incorporated.

However, the International Committee on Systematics of Prokaryotes' (ICSP) Subcommittee on the taxonomy of Mollicutes reviewed the work by Gupta et al. during its 2018 meeting, and published a paper recommending the rejection of the new classification (Balish et al., 2019). In that paper, the authors specifically stated: "the consensus opinion of the Subcommittee members is that the proposed nomenclatural revisions are at the present time an unnecessary over-reach verging on taxonomic vandalism. It is highly doubtful the nomenclature proposed will ever be adopted, either on practical grounds involving the names of major pathogens currently regulated in medicine and agriculture by international laws, or by the community of specialists". For this reason, and following the ICSP recommendations, this PhD thesis will not follow the taxonomic reclassifications proposed by Gupta et al. (2018).

1.2 Porcine mycoplasmas

To date, six Mycoplasma species have been identified in pigs. *Mycoplasma hyopneumoniae* (Goodwin et al., 1965; Mare & Switzer, 1965) causes enzootic pneumonia in finishing pigs. *Mycoplasma hyorhinis*, the first mycoplasma isolated from swine (Switzer, 1955), is a pathobiont bacterium, as it is ubiquitous in the respiratory tract of healthy pigs and can also cause polyserositis (Friis & Feenstra, 1994), arthritis (Martinson et al., 2018), otitis (Morita et al., 1995) and conjunctivitis (Resende et al., 2019). *Mycoplasma hyosynoviae* (Ross & Karmon, 1970), another pathobiont of swine, primarily colonizes the tonsils at young age and persists as pigs age, sporadically causing non-suppurative arthritis in growing and adult pigs. *Mycoplasma suis* (Splitter, 1950), an hemotropic mycoplasma, causes anemia in pigs by attaching to and lysing erythrocytes. *Mycoplasma flocculare* (Friis & Meyling, 1972) and *Mycoplasma hyopharyngis* (Erickson et al., 1986) are commensal, non-pathogenic inhabitants of the upper respiratory tract of normal pigs.

1.3 ***Mycoplasma hyopneumoniae***

1.3.1 *Etiology and diversity*

Although *M. hyopneumoniae* was first identified as the cause of swine enzootic pneumonia (EP) in 1965, descriptions of a disease characterized by chronic cough and protracted loss of condition in pigs – presumably EP - were first reported in the 1930s. Köbe (1933) referred to this respiratory condition as Ferkelgrippe, and Glässer (1939) as Schweinegrippe, that is, piglet or pig flu, respectively, in German. It was not until 1951 that the chronic respiratory condition caused by *M. hyopneumoniae* was recognized as a separate disease entity from swine influenza and thus, it was termed parainfluenza or infectious pneumonia. This differentiation was based on a series of observations by Gulrajani & Beveridge (1951) of outbreaks of pneumonia in the United Kingdom, in which swine influenza virus could not be isolated from pigs and recovered pigs harbored no antibodies against influenza virus. Additionally, when naïve pigs were exposed to bacteria-free filtrates from pneumonic lung, the onset of disease was slower and the course of infection more protracted compared to typical swine influenza.

Despite influenza virus being rejected as the causative agent of EP, the disease was still referred to as virus pneumonia, influenza-like pneumonia or enzootic virus pneumonia, as a viral etiology was suspected. This was based on a study by Betts & Beveridge, 1952 in which the transmissible agent could pass through a bacteriological filter, therefore indicating (with the knowledge of that time) that it was a virus. However, the agent was also found to be sensitive to tetracycline, this perhaps being an early clue that the agent was, in reality, not a virus.

The identification of a bacterial etiology in swine EP was recognized in 1965, when Mare and Switzer isolated a microorganism from a case of EP in the USA both in broth and in solid medium, and used it to reproduce the characteristic lesions of the disease. These new bacterial colonies were named *M. hyopneumoniae* (Mare & Switzer, 1965). Two months later, Goodwin and collaborators published the isolation of the causative organism of EP cases in the United Kingdom and named it *M. suipneumoniae* (Goodwin et al., 1965). At first, there was uncertainty whether these were two different pathogens, but this was resolved when both were shown to be serologically identical by growth-inhibition and metabolic-inhibition tests (Goodwin et al., 1967). The name *M. hyopneumoniae* prevailed over *M. suipneumoniae* in future publications and it remained unchanged until nowadays.

Mycoplasma hyopneumoniae strains circulate globally in swine herds, and variability across several strains and isolates has been demonstrated by antigenic, proteomic, transcriptomic, genomic and virulence level characterization (reviewed by Betlach et al., 2019). However, no standardized methods for characterizing strains are currently in place for this pathogen, and the lack of a defined, broadly-agreed upon terminology hinders the ability to compare published results. Efforts at establishing a defined terminology have been recently made, since the usage of typing methods (mostly Multiple-Locus Variable number tandem repeat Analysis, MLVA) and sequencing data for *M. hyopneumoniae* molecular characterization has surged due to their potential application in control and elimination strategies. In that sense, the establishment of a shared database that includes partial or complete sequencing data for *M. hyopneumoniae* field strains would be beneficial in epidemiological investigations aiming at understanding strain dissemination and elimination failures.

1.3.2 *Pathogenesis*

The capacity of a microbe to cause damage in a host is defined as pathogenicity (Casadevall, 2017). Such damage is mostly defined as the manifestation of clinical signs, which occur only when the host-microbe interactions result in sufficient host deterioration to disrupt homeostasis, usually through the expression of virulence factors by the microbe (Casadevall & Pirofski, 1999).

Mycoplasma hyopneumoniae is an exclusive swine pathogen, capable of replicating and spreading in infected pigs and causing clinical disease and lesions. Although the pathogenesis is not fully understood, still with several knowledge gaps (Maes et al., 2018), a sequence of events including colonization and adherence to ciliated respiratory epithelial cells, impairment of the mucociliary apparatus, and modulation of the host immune responses need to occur for an infection to be established (Blanchard et al., 1992; Kwon et al., 2002).

In order to initiate colonization of the respiratory mucosa, inhaled *Mycoplasma hyopneumoniae* cells have to overcome the primary innate immune defense system of the mammalian respiratory tract, namely the mucociliary clearance system (Knowles & Boucher, 2002). This system is mainly composed of stratified mucus secreted by the respiratory goblet cells, which covers the ciliated epithelial cells and enables the synchronic beating of cilia to expel the mucus containing microorganisms and foreign particulate matter upstream of the airways, to be ejected by coughing, sneezing or swallowing (Bustamante-Marin & Ostrowski, 2017). It is not known how *M. hyopneumoniae* penetrates the thick mucus layer covering the cells, but it is known that adherence to ciliated epithelial cells is a requisite for infection. A repertoire of at least 35 *M. hyopneumoniae* proteins have been associated with cell adhesion, most notably members of the P97/P102 paralog family of adhesins (Hsu et al., 1997). These adhesion proteins undergo maturation by posttranslational cleavage, resulting in a diverse array of fragments that bind multiple host ligands in the cell surface or the extracellular matrix, such as plasminogen, fibronectin or heparin (Raymond et al., 2015; reviewed by Leal Zimmer et al., 2020). Electron microscopic studies have revealed that adhesion occurs between the cilia and the microvilli of epithelial cells (Tajima & Yagihashi, 1982), which induces ciliostasis, loss of cilia and eventual degeneration and necrosis of epithelial cells (DeBey & Ross, 1994). Additionally, the presence of *M. hyopneumoniae* in the respiratory tract is sensed by different families of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). Toll-like receptor 2 and TLR6 have been described to be involved in the recognition of *M. hyopneumoniae* by porcine alveolar macrophages (Muneta et al., 2003). After bacterial sensing, the host immune response is activated and inflammatory cytokines such as IL-1 and TNF-α are produced, stimulating the influx of inflammatory cells, mainly neutrophils and macrophages (Kwon et al., 2002). In fact, the host inflammatory response is responsible for the induction of pulmonary lesions and consequently, the development of clinical signs.

The course of the infection with *M. hyopneumoniae*, as with other respiratory infections caused by *Mycoplasma* species, is chronic by definition (Dobbs et al., 2009). Thus, it is evident that the efficiency of the immune system is imperfect at clearing the pathogen from the respiratory system. This might be associated with different mechanisms of immune evasion elicited by *M. hyopneumoniae* cells. Although alveolar macrophages, neutrophils and mucosal resident histiocytes are the main cell types responsible for engulfing and destroying invading susceptible microorganisms (Murphy et al., 2012), *M. hyopneumoniae* can evade phagocytic uptake by porcine alveolar macrophages *in vitro*, perhaps explaining why bacteria are not quickly cleared by the respiratory immune system (Deeney et al., 2019). Additionally, *M. hyopneumoniae* has been shown to interact with superficial actin on epithelial cells, causing cytoskeletal arrangements that promote bacterial endocytosis (Raymond, Madhkoor, et al., 2018). Once endocytosed, small proportions of the bacterial population can survive within the endosomes, as well as escape them and reside intracellularly within the cytoplasm (Raymond, Turnbull, et al., 2018). Yet another study, using an air-liquid interface culture system for porcine bronchial epithelial cells, described the ability of *M. hyopneumoniae* to disrupt the epithelial barrier and migrate to the basolateral chamber through the paracellular route (Wang et al., 2020). Although these mechanisms have, so far, been only demonstrated *in vitro*, they could potentially not only contribute to evasion from the immune system but also allow the dissemination of the pathogen to organs outside the respiratory system, as well as contribute to its persistence within the pig without causing clinical signs. In fact, several experimental *in vivo* studies appear to support the existence of such mechanisms. Pieters et al. (2009) showed that *M. hyopneumoniae* persisted in the lungs for long periods, at least 214 days, and that infected pigs remained contagious to others during at least 200 days, which was long after disease recovery. Bacterial isolation of *M. hyopneumoniae* from extra-respiratory tissues such as the liver, spleen, kidney and brain has been described in a limited number of pigs that were inoculated through the respiratory route under experimental conditions (Le Carrou et al., 2006; Marois et al., 2007). In other instances, *M. hyopneumoniae* DNA has been detected in the same tissues after experimental infection with a particular field strain (Woolley et al., 2012), as well as in body fluids distant from the respiratory system, such as in piglet processing fluids (Vilalta et al., 2019, 2020) and boar semen (Milovanovi et al., 2017). Thus, it seems plausible that *M. hyopneumoniae* cells are mechanistically capable of disseminating systemically, at least transiently and for some particular strains. However, in the latter examples, the viability of the detected genetic material could not be ascertained, raising questions on the true significance of the findings, as it is currently unknown if, and for how long, the genetic material from non-viable *M. hyopneumoniae* cells persists in the host and remains detectable by PCR. Thus, to gain further knowledge on the pathogenesis of *M. hyopneumoniae*, research aiming at developing techniques to determine bacterial viability is needed. Such research may also contribute to improved diagnostic capabilities for this pathogen, which could be used in disease prevention and control strategies.

1.3.3 *Epidemiology*

Infections caused by *Mycoplasma hyopneumoniae* are considered to affect pigs worldwide, although prevalence data is scarce since it is not a reportable disease (Pieters & Maes, 2019). However, the implementation of programs aiming at eradicating the pathogen from pig populations are on the rise (Yeske et al., 2020), and have been completed either at regional or national levels, such as in Switzerland (Stärk et al., 2007), Norway (Gulliksen et al., 2021) and Finland (Rautiainen et al., 2001).

1.3.3.1 *Production and animal welfare impact of M. hyopneumoniae infection*

Production and animal welfare losses attributed to *M. hyopneumoniae* infections, both as a primary agent and in co-infections with other respiratory pathogens, occur mostly in the grow-finishing phase of production. Economic expenses associated with disease occurrence include increased antibiotic usage and vaccine application, reduction in growth rates, increased feed conversion, mortality and culling rates. Several studies have estimated productivity losses and other economic impacts due to *M. hyopneumoniae* infections under field conditions. A recent study (Silva et al., 2019) compared groups of finishing pigs sourced from either *M. hyopneumoniae*-positive or negative sow farms. In pigs from positive sow farms, there was a 4.2% reduction in growth rate, a 0.6% increase in feed conversion and a 24.5% increased mortality rate compared to negative pigs. The authors also estimated an increased cost of $1.20 in antibiotic treatments and $0.25 in vaccination cost per marketed pig in the *M. hyopneumoniae*-infected groups. Taken together, *M. hyopneumoniae* infection was estimated to increase the cost of production by an average of US$7.00 per pig marketed. The economic impact has been estimated to be even higher, of US$9.69 and US$10.12 per marketed pig if *M. hyopneumoniae* is in combination with PRRSV or IAV-S, respectively (Dykhuis-Haden et al., 2012).

Under field conditions, the occurrence of respiratory infections caused solely by *M. hyopneumoniae* are uncommon. Most times, multiple pathogens circulate within a herd, leading to co-infections of multiple etiologies. The term ‘Porcine respiratory disease complex (PRDC)’ is used to describe the clinical respiratory condition in which various bacterial and viral pathogens interact to express disease (Opriessnig et al., 2011). Indeed, *M. hyopneumoniae* is considered a primary pathogen in PRDC, as it plays a ‘door opener’ role by disrupting the mucociliary clearance apparatus and allowing pathogens and commensal bacterial of the upper respiratory tract to colonize and establish in the lungs. Several studies, reviewed by Marois-Créhan et al. (2020) have shown that *M. hyopneumoniae* interacts with various respiratory bacteria and viruses, most often resulting in synergistic or additive effects in clinical signs or lung lesion severity, indicating that diminishing the detrimental effects of *M. hyopneumoniae* infections can help reduce the impact of infections caused by other pathogens.

1.3.3.2 *Transmission*

Mycoplasma hyopneumoniae is principally transmitted directly through nose-to-nose contact between infected and susceptible pigs, as a study showed that the risk for infection between pigs sharing the same airspace by direct contact was seven times higher than by indirect contact (Morris et al., 1995). However, indirect transmission through aerosols has been suggested, as proximity to other farms or slaughterhouses has been associated with a higher risk of *M. hyopneumoniae* infection (Goodwin, 1985). Aerosolized *M. hyopneumoniae* has been detected and confirmed to be infectious up to 9.2 km from a positive farm through the collection of air samples and a bioassay (Otake et al., 2010). Contrarily, indirect transmission through the spread of infected respiratory secretions on fomites, personnel and environmental surfaces, although plausible, has been deemed of little importance. This is based on a 20-week period study in which the bacterium was not transmitted by farm personnel that moved weekly from an infected to an uninfected farm and followed standard hygiene protocols (Batista et al., 2004). In addition, the scarcity of studies regarding indirect transmission might be in part due to the intrinsic difficulty to determine viability -and thus, potential infectiveness- of this bacterium, as it is known to be extremely difficult to grow in culture, and also due to the limited ability to utilize environmental substracts for metabolism that may compromise its survivability outside the pig's respiratory tract.

Nevertheless, there is mounting evidence that *M. hyopneumoniae* can remain viable outside the host for a relevant amount of time. Goodwin (1972) was the first to investigate the survival time of *M. hyopneumoniae* in liquid medium, solid medium and in small pieces of pneumonic tissue, determined by the subsequent recovery of the organism in liquid medium. In his experiments, *M. hyopneumoniae* survived in liquid medium at room temperature for 18 to 31 days, and in the refrigerator (between 4°C and 11°C) for 7 to 14 weeks. On solid medium incubated at 37°C the survival time was at least 20 days and, in lung tissue, the survival time was 7 days at room temperature and at least 11 days in the refrigerator. Similarly, Friis (1973) showed that different swine mycoplasmas could survive for various days after air-drying and storage at room temperature. In the case of *M. hyopneumoniae,* the length of time that the bacterium could remain desiccated before successful re-culture was at least 4 days. In a more recent *in vitro* study conducted by Browne et al. (2017) using different *M. hyopneumoniae* strains, the time of survival outside the host was extended to up to 8 days, especially in conditions of low temperature and presence of dust. The authors assessed the above-mentioned conditions using different types of surface materials usually present in swine farms.

The findings mentioned above suggest that, at least under controlled conditions in laboratory settings, the resilience of *M. hyopneumoniae* to survive outside the respiratory tract of pigs is not to be ignored. However, the development of culture-independent methods to determine the viability of *M. hyopneumoniae* is needed in order to obtain more accurate information, since, as already stated, bacterial culture is known to have very low sensitivity.

With improved diagnostic tools at hand, it would be a logical next step to design studies aiming at assessing the risk factors and housing conditions that could be associated with the indirect transmission of *M. hyopneumoniae*. With that information, internal biosecurity measures such as cleaning and disinfection would potentially need to be refined.

1.3.3.3 *Infection dynamics*

The dynamics of *M. hyopneumoniae* infections, highlighting the key events that occur throughout the different stages of the infection process, have been recently reviewed by Garcia-Morante et al. (2022).

Infections with *M. hyopneumoniae* are frequently divided into two phases, acute and chronic, based on the presentation of clinical signs (Pieters et al., 2009; Sibila et al., 2009). The minimal dose to induce infection in pigs under experimental conditions has been determined to be 1 x 10^5 color changing units (CCU) per pig (Marois et al., 2010). The acute phase of infection starts immediately after inoculation, and *M. hyopneumoniae* can be detected by PCR in *ante-mortem* samples five days post-inoculation (Pieters et al., 2017). However, detection of the bacterium by PCR was not obtained in *ante-mortem* samples, including tracheobronchial lavage fluid, up to two days post-inoculation in the same experimental study. A true estimation of the latent period for *M. hyopneumoniae*, the time interval between infection and infectiousness (transmission to other pigs), has not been established. The incubation period has been estimated to be between 10 and 16 days in experimental conditions, with coughing peaking three to five weeks post-inoculation (Sørensen et al., 1997; Arsenakis et al., 2016). Pneumonia may start developing one week after infection (Lorenzo et al., 2006; Underdahl et al., 1980), increasing progressively until reaching maximal extension four weeks post-infection (Garcia-Morante et al., 2017). A chronological, clear-cut distinction between acute and chronic infection has not been established for *M. hyopneumoniae* and there is most likely a transition between acute, subacute and chronic stages of disease development. Certainly, the chronic period is the most extended and diagnostically challenging. Coughing gradually disappears and inoculated pigs commonly cease coughing between 8- and 14-weeks post-infection, while lung lesions are generally resolved 12 weeks post-infection, with interlobular scarring and lung parenchymal retraction as residual macroscopic lesions (Kobisch et al., 1993; Sørensen et al., 1997). Thus, the bulk of the infection period in general, and of the chronic phase in particular, is characterized by an asymptomatic carrier state, as pigs may remain chronically infected for several months (Fano et al., 2005; McDowell et al., 2023; Pieters et al., 2009).

The determination of infection clearance post-inoculation, or pathogen persistence in the host, usually encompasses PCR detection of *M. hyopneumoniae* DNA, either in *ante-mortem* or *post-mortem* samples. Current PCR assays for *M. hyopneumoniae* detect DNA derived from live and dead bacterial cells and thus, cannot be used to identify viable (and thus, potentially infectious) cells or to assess the metabolic activity of the detected genetic material (Lauri & Mariani, 2009). Indeed, it is conceptually possible that positive PCR results at final stages of chronic infections may represent the mere presence of residual DNA from non-viable *M. hyopneumoniae* cells that have not been cleared from the respiratory tract. This diagnostic uncertainty was evidenced in a recent study in which a longer than expected persistence of *M. hyopneumoniae* detection by PCR post-exposure was described, although the viability of the detected genetic material could not be ascertained (McDowell et al., 2023). Therefore, the inability to assess bacterial viability is a key limiting factor for the diagnosis of *M. hyopneumoniae*, especially when evaluating the efficacy of disease control interventions in the field, such as pathogen eradication, that rely on the duration of pathogen persistence post-infection.

1.3.4 *Control and elimination*

1.3.4.1 *Animal management practices*

Several factors can influence the transmission patterns of *M. hyopneumoniae* both between and within herds, as well as the clinical manifestation of infection. Parameters such as pig density, herd size, and health status of replacement animals are among the most prominent, and thus, efforts at modifying production, management and housing conditions are important for disease control (Marco et al., 2020).

Even though the effects of *M. hyopneumoniae* infection are evident in the grow-finishing period, the health status of the source sow herd is of paramount importance for the development of the disease in downstream flows (Pijoan, 2005). This was evidenced by a study by Fano et al., 2007 in which a positive correlation between the pre-weaning prevalence of *M. hyopneumoniae* infection and disease severity in finishing pigs was observed. Thus, the swine industry has widely agreed to focus on the breeding herd to control the infection, and diagnostic challenges have been principally associated with the introduction of replacement gilts in two main ways. Firstly, in infected sow herds, the introduction of high health replacement gilts can be problematic as they may serve as biological reservoirs for continued pathogen transmission and subpopulation formation (Takeuti et al., 2023), contributing to whole-herd instability (Pieters & Fano, 2016). Therefore, gilt acclimation strategies such as vaccination (Garza-Moreno et al., 2018) or early controlled exposure with lung inocula containing *M. hyopneumoniae* in order to create immunity before entering the sow herd (Robbins et al., 2019), have been described. Secondly, in naïve sow herds, replacement gilts need to be introduced in the herd only after their negative status for *M. hyopneumoniae* has been established.

In these cases, refined diagnostic testing strategies, specifically aiming at increasing detection sensitivity in low prevalence scenarios, are needed (Betlach et al., 2021; Sponheim et al., 2021). Furthermore, additional diagnostic capabilities could be applied to such situations. For example, having a diagnostic assay to determine the viability and metabolic activity of the *M. hyopneumoniae* cells present in the lung inocula used for directed exposure would be beneficial, since failures at obtaining homogeneous infection of pig populations post-exposure may occur without apparent cause. Another example could be having a diagnostic assay that could serve as a confirmatory test for the gilts that test positive by PCR (usually with high Ct values), for which the true nature of the genetic material that is being detected is uncertain.

1.3.4.2 *Vaccination*

Vaccination of susceptible pig populations, such as weanling piglets and replacement gilts, is very frequently used for *M. hyopneumoniae*, as it improves the health and performance of the animals and reduces the use of antimicrobials. Commercial vaccines consist of inactivated, whole-cell preparations (bacterins) with different adjuvants and are administered intramuscularly (Maes et al., 2018, 2021). The exact mechanisms of protection are not fully understood, as even if *M. hyopneumoniae*-specific antibodies are detected in BALF (Marchioro et al., 2013) and serum (Sibila et al., 2004) after vaccination, it is generally accepted that antibody concentrations do not directly correlate with protection against infection (Djordjevic et al., 1997; Thacker et al., 1998).

There are multiple advantages associated with piglet vaccination, such as improved daily weight gain, feed conversion ratio and carcass homogeneity, and reduced clinical signs, lung lesions and treatment costs (Maes et al., 1999; Maes et al., 1998). Vaccination also reduces the number of *M. hyopneumoniae* cells in the respiratory tract (Vranckx et al., 2012) and decreases the herd infection levels (Sibila et al., 2007). This level of protection is, however, incomplete, and vaccination does not prevent colonization or significantly reduces the rate of transmission of *M. hyopneumoniae* (Pieters et al., 2010; Villarreal et al., 2011). Nevertheless, a recent study showed that vaccination, when coupled with antibiotic treatment, was associated with a numerical reduction of the relative bacterial load, compared to untreated, unvaccinated pigs (Betlach et al., 2021). Thus, further research is needed to understand the effect that vaccination has on the epidemiology of the disease.

1.3.4.3 *Antibiotic treatment*

For *M. hyopneumoniae* infections, antibiotic treatment is needed when clinical disease exists or is anticipated in order to prevent economic losses due to decreased performance, and to protect animal welfare and wellbeing. Similar to vaccination, antibiotic medication can help at reducing the clinical signs, lesions and performance impact associated with *M. hyopneumoniae* infections (Goodwin, 1979) without preventing bacterial colonization. Tetracyclines and macrolides are specifically used to control and treat *M. hyopneumoniae* infections, since mycoplasmas are intrinsically resistant to beta-lactams, glycopeptides, polymyxins, sulfonamides, trimethoprim, rifampicin, first generation quinolones and 14-membered lactone ring macrolides such as erythromycin (Gautier-Bouchardon, 2020).

Several studies have been conducted to assess the efficacy of various antibiotics against *M. hyopneumoniae* infections under experimental and field conditions (Gautier-Bouchardon, 2020). Efficacy has been evaluated using production (average daily weight gain, feed conversion) or clincal-pathologic (coughing index, lung lesion scoring) parameters, and for the majority of antibiotics tested, these parameters improved after treatment. However, if persistence of infection or ability to clear *M. hyopneumoniae* cells post-treatment are used as parameters to measure antibiotic efficacy, several studies evidenced that various antibiotics may not be efficacious. Persistence of detection and/or isolation of *M. hyopneumoniae* has occurred after administration of tetracyclines (Huhn, 1971; Thacker et al., 2006), macrolides (Painter et al., 2012; Vicca et al., 2005), fluoroquinolones (Le Carrou et al., 2006) or pleuromutilins (Ross & Cox, 1988), among others. However, the interpretation of PCR detection of *M. hyopneumoniae* can be specially challenging in the context of antibiotic treatment, as PCR assays for *M. hyopneumoniae* are based on bacterial DNA detection, and thus, the viability of the detected genetic material cannot be ascertained (Josephson et al., 1993). Hence, PCR may not be an ideal method to evaluate treatment efficacy, as a positive result may represent nothing more than the detection of residual, non-cleared genetic material from non-viable cells. A good example of this situation is described in a study by Betlach et al. (2021), in which, in tulathromycin-treated gilts, *M. hyopneumoniae* genetic material was detected by PCR up to approximately three months after receiving antibiotic treatment. However, transmission of the bacterium from these persistently infected pigs to close-contact, naïve pigs was not shown, suggesting that PCR results could potentially represent the detection of non-viable cells.

The aforementioned studies highlight that, in order to evaluate the efficacy of antibiotic treatments against *M. hyopneumoniae*, it is essential to determine bacterial viability, although the remarkably low sensitivity of bacterial culture has been a limiting factor in this context (Friis, 1975). Thus, culture-independent methods for assessing *M. hyopneumoniae* viability need to be developed and applied in infected pigs pre- and post-treatment, to be used as indications of antibiotic treatment efficacy.

1.3.5 *Diagnostics*

Accurate diagnostics are the cornerstone in which disease prevention and control interventions depend on. Every diagnostic investigation should start with two simple questions: “what information do I need to obtain?” and “how will I interpret and use the obtained information?”. In order to answer the first question, understanding *M. hyopneumoniae* infection dynamics, collecting appropriate specimens at specific times, and choosing the right laboratory diagnostic tools are key to obtaining reliable diagnostic results (reviewed by Garcia-Morante et al., 2022). For the second question, and even though test result interpretation is usually straightforward, one should be prepared to face unexpected results that require further investigation, confirmation through parallel or serial testing, or expert opinion consultation.

1.3.5.1 *Clinical-pathologic diagnosis*

Mycoplasma hyopneumoniae infections manifest clinically as intermittent, non-productive cough of variable severity in growing-finishing pigs. Additionally, and especially in uncomplicated cases, infections can remain subclinical with pigs showing no clinical signs (Fano et al., 2005). Contrarily, outbreaks in naïve pig populations can course with dyspnea, pyrexia, anorexia, lethargy and even death, especially when combined with other pathogens (Bargen, 2004). Importantly, pigs can still shed the pathogen and be infectious to others even when clinical signs have long resolved, in chronic stages of infection (Pieters et al., 2009).

Pathological examination of pigs showing clinical signs compatible with an infection with *M. hyopneumoniae* should be included in a comprehensive disease investigation. Macroscopically, characteristic lung lesions induced by *M. hyopneumoniae* consist of red-tan-gray tissue discoloration, collapse and rubbery firmness, mainly affecting the cranioventral regions of the lungs in a lobular pattern (Caswell & Williams, 2016), a lesional pattern usually referred to as cranioventral pulmonary consolidation (CVPC; Fraile et al., 2010). Lung lesion extension is usually correlated to clinical signs at herd level, and evaluation at necropsy or at slaughter to obtain information from implemented control measures is a common practice in Europe, usually using an array of different macroscopic scoring systems (Garcia-Morante et al., 2016). The potential implementation of artificial intelligence-based technologies in macroscopic lung lesion evaluation may reduce subjectivity and inter-observer bias and potentially be used as routine disease monitoring tools (Maes et al., 2023).

The histologic evaluation of lung lesions is particularly useful in *M. hyopneumoniae* infections since, apart from revealing characteristic lesions of the infection and differentiating them from the ones caused by other respiratory pathogens, it can allow visualization of incipient lesions not yet observable at macroscopic level or reveal healing responses, indicative of chronic infection. *Mycoplasma hyopneumoniae*-associated lesions are described as broncho-interstitial pneumonia, with characteristic hyperplasia of the bronchus-associated lymphoid tissue (BALT), which severity can be assessed employing different scoring systems (Calsamiglia et al., 2000; Livingston et al., 1972; Morris et al., 1995; Woolley et al., 2012). One of the main pitfalls of some of these systems is that severity is related only to the extension of BALT hyperplasia. Hyperplasia of BALT is intrinsically associated with the chronicity of lesions, and often remains residual when the cellular exudate has been cleared. Hence, high microscopic scores should be characterized not only by extensive peribronchiolar and perivascular lymphoid hyperplasia, but also by the abundance of inflammatory infiltrates, such as in the scoring system performed in Woolley et al. (2012).

The onset, duration and severity of clinical signs and lung lesions may be influenced by the virulence of the circulating *M. hyopneumoniae* strain (Vicca et al., 2003; Woolley et al., 2012), the number of different strains/variants circulating in the farm (Michiels et al., 2017), and the coexistence of other respiratory pathogens in the context of PRDC (Saade et al., 2020). Therefore, given the lack of pathognomonic characteristics of *M. hyopneumoniae* infections, particularly of the clinical signs, both clinical and pathologic diagnoses need laboratory confirmation.

1.3.5.2 *Detection of antibodies*

The induction of an antibody-producing, humoral response against *M. hyopneumoniae* infections is slow, inconsistent between pigs, and seroconversion is chronologically variable, as pigs within a population may get infected at different moments (Poeta Silva et al., 2020; Sibila et al., 2009). These limitations, coupled with the frequent interaction with concurrent herd vaccination programs, make serological diagnoses challenging and thus, antibody-based assays are usually utilized in combination with other diagnostic tools (Gomes Neto et al., 2014; Maes et al., 2018). The detection of antibodies is mainly performed by enzyme-linked immunosorbent assays (ELISA) specifically targeting anti-*M. hyopneumoniae* immunoglobulin G (IgG) isotype (Sibila et al., 2009). Commercial assays are designed to detect antibodies in serum, but some have been modified and validated for use in BALF, oral fluids and other tissue fluids (Morris et al., 1995; Pieters et al., 2017). Different studies have compared the sensitivity and specificity of commercially available ELISA assays. The results highlighted that blocking ELISA assays are more sensitive, as they showed earlier onset of antibody detection compared to indirect ELISA assays (Fano et al., 2012). Additionally, in terms of specificity, it was shown that cross-reactivity with *M. hyosynoviae*, *M. hyorhinis* and *M. flocculare* can occur in some assays (Erlandson et al., 2005; Gomes Neto et al., 2014; Poeta Silva et al., 2020).

Seroconversion has been reported to first occur as early as 7-9 days post-infection in experimental conditions (Sørensen et al., 1997). However, the onset is most frequently described to be at 2-3 weeks post-infection (Pieters et al., 2017; Poeta Silva et al., 2020) and even later in field conditions (Fano et al., 2005; Pieters et al., 2009). Thus, it can be inaccurate (and risky) to determine infection status for *M. hyopneumoniae* based on serological assay results, as pigs can be infected and shedding the bacterium long before having detectable antibodies circulating. Serological diagnoses, however, can be useful to monitor infection progression (collecting paired serum samples) or to evaluate responses to vaccination (Maes et al., 2021).

1.3.5.3 *Detection of the pathogen*

1.3.5.3.1 *Detection of antigens*

Specific antigenic proteins of *M. hyopneumoniae* can be detected in tissues via immunofluorescence (Amanfu et al., 1984) and immunohistochemistry (Opriessnig et al., 2004; Sarradell et al., 2003). The usefulness of these techniques relies on the visualization of the targeted pathogen in physical proximity to the characteristic lesions they produce, providing evidence of their causative role in diseases. However, some limitations prevent these techniques from being employed routinely in diagnostic investigations and thus, they have been mainly utilized for research purposes. Disadvantages include that these assays can only be performed in *post-mortem*, formalin-fixed paraffin-embedded tissues, and that specificity and sensitivity highly depend on the quality of the sample and the type of antibody employed. While monoclonal antibodies have higher specificity because they target a single epitope, polyclonal antibodies tend to have a higher sensitivity as they target more epitopes, increasing the antigen-antibody binding and the overall immunostaining signal.

The popularity of point-of-care immunoassays, based on the rapid detection of antigens, has been on the rise recently, probably due to their extensive use in human diagnostics of SARS-CoV-2. However, to date, this approach has been poorly explored in swine, has mainly been described for virus detection (Goodell et al., 2013; Li et al., 2022), and has not been reported for *M. hyopneumoniae*.

1.3.5.3.2 *Bacterial isolation*

Bacterial isolation has been historically considered the gold standard technique to determine the presence of *M. hyopneumoniae* in diagnostic samples (Sibila et al., 2009; Thacker, 2004). However, *M. hyopneumoniae* is a very fastidious bacterium to grow in culture, with isolation rates as low as 2.6% (Friis, 1971), mostly due to the highly specific nutritional requirements and the optimal incubation conditions needed (Friis, 1975; Kobisch & Friis, 1996). Therefore, a negative culture result should never be a conclusive test, as it does not exclude the presence of *M. hyopneumoniae* in a sample, and thus, perhaps other molecular detection techniques should become the gold standard method.

Because of its low sensitivity, bacterial isolation is not routinely performed for diagnostic purposes, but isolation may be required for vaccine development (especially autogenous vaccine), antimicrobial susceptibility testing, or molecular epidemiology studies, among others. Hence, research aiming at identifying the optimal growth conditions for *M. hyopneumoniae* has been performed. Several modifications to the Friis medium, the characteristic liquid broth containing fresh yeast extract, brain heart infusion broth, thallium acetate, swine and horse serum and phenol red as a pH indicator used for *M. hyopneumoniae* isolation have been applied, although their detailed formula is sometimes commercially protected (Cook et al., 2016). Some of these modifications include the addition of specific amino acids and/or peptone and other cofactors (Ferrarini et al., 2016), while others have the objective of preventing the frequent contamination and overgrowth of *M. hyorhinis*, and include the use of 5% hyperimmune anti-*M. hyorhinis* serum together with cycloserine addition (Kobisch & Friis, 1996), or the addition of kanamycin (Cook et al., 2016).

Despite all the aforementioned limitations, bacterial culture remains the only method currently available to determine *M. hyopneumoniae* viability. This fact has important implications, not only in diagnostic instances, but also from the point of view of better understanding the pathogenesis and the epidemiology of the disease. Determining the viability of *M. hyopneumoniae* is essential to, for example, defining the biological relevance of the extrapulmonary detection of the bacterium, to assess the efficacy of antibiotic treatments, or to characterize the clearance of infection at late stages of the chronic phase of infection, among others. Therefore, the development of diagnostic methods to assess the viability of *M. hyopneumoniae* that do not rely in bacterial culture are desperately needed. If obtained, their applicability in different aspects of disease diagnosis and epidemiology could be significant.

1.3.5.3.3 *Detection of nucleic acids*

The detection of *M. hyopneumoniae* in clinical samples is most frequently accomplished by using polymerase chain reaction (PCR) assays targeting bacterial nucleic acids, both for research and diagnostic purposes.

Several PCR assays have been developed and applied to detect *M. hyopneumoniae* in clinical specimens reviewed by Garcia-Morante et al. (2022). Conventional PCR assays were first introduced in the early 1990s, and the use of a single set of primers appeared to lack sufficient sensitivity (Artiushin et al., 1993; Baumeister et al., 1998; Blanchard et al., 1996; Harasawa et al., 1991; Stemke et al., 1994). Thus, highly sensitive nested PCR assays were later developed (Calsamiglia et al., 1999; Kurth et al., 2002; Stärk et al., 1998; Stemke, 1997; Verdin et al., 2000). These assays are based on two primer specific amplifications of the *M. hyopneumoniae* nucleic acid, using internal specific primers complementary to the nucleotide sequence of the first amplification. However, such high sensitivity is commonly associated with problems related to cross-contamination and potential false positive amplifications. Importantly, both conventional and nested PCR techniques generate qualitative (positive/negative), visually estimated outcomes.

In the 2000s, species-specific, real-time PCR assays for *M. hyopneumoniae* were developed (Dubosson et al., 2004; Marois et al., 2010; Strait et al., 2008) and have become the preferred laboratory technique for *M. hyopneumoniae* detection. Real-time PCR assays have several advantages over their preceding counterparts. First, since the amplification of the targeted DNA molecule(s) is monitored via a fluorescent reporter in real time and not at the end of the reaction, the generated outcome is a semiquantitative value of the amount of genetic material originally present in the sample. Quantitative values can be also established by obtaining absolute genome copy numbers extrapolated from a standard curve, constructed with dilutions of a positive control of known concentration. Second, sensitivity and specificity can be highly improved by using probe-based chemistry (in contrast to SYBR green), in which sequence-specific DNA probes are labeled with a fluorescent dye, which permits detection only after hybridization of the probe with its complimentary sequence. Third, real-time PCR systems enable the use of reference genes as internal controls for extraction and amplification steps, as well as for data normalization. Lastly, real-time PCR assays for *M. hyopneumoniae* can be designed to detect one or more gene targets of the same pathogen (monoplex) or of different pathogens (multiplex; (Fourour et al., 2018; Lung et al., 2017)).

Additionally, a digital droplet PCR assay that allows absolute quantification of the DNA copy number of *M. hyopneumoniae* has been recently developed (Beuckelaere et al., 2022).

The overall sensitivity of PCR detection of *M. hyopneumoniae* can be influenced by several factors, such as the sample collection material, the nucleic acid extraction method, the sample type, and the chronicity of the infection. Takeuti et al., (2017) showed that nylon-flocked swabs had higher absorption capacity and higher rates of *M. hyopneumoniae* detection than rayon-bud swabs. Vangroenweghe et al. (2015) reported better PCR detection of *M. hyopneumoniae* when samples were extracted with a total nucleic acid extraction kit, compared to a viral RNA extraction kit.

Several sample types, including clinical specimens collected *ante*- or *post-mortem* and environmental samples have been used for the PCR detection of *M. hyopneumoniae*. In general, suitable samples for *M. hyopneumoniae* detection represent different parts of the respiratory conductive system, as they are lined by ciliated epithelial cells in which the bacterium attaches. Various terms to indicate the sample types are used in the literature, such as nasal, laryngeal, tracheobronchial, tracheal and bronchial swabs, and deep tracheal catheters. All of these terms relate to the material used to collect the sample (swabs or catheters) rather than the actual specimen that is collected for sampling. Thus, in this document the word ‘secretions’ will be used to refer to the sample type (i.e. nasal secretions, tracheal secretions, etc.).

Samples obtained from deeper parts of the respiratory airways provide the highest diagnostic sensitivity, in both experimentally and naturally infected pigs. Thus, bronchial secretions and broncho-alveolar lavage fluid samples (obtained *post-mortem*) provide the highest overall sensitivity, while tracheal secretions, followed by laryngeal and finally nasal secretions samples are the most sensitive sample types obtained *ante-mortem* (Betlach et al., 2020; Clavijo et al., 2021; Fablet et al., 2010; Moiso et al., 2020; Pieters et al., 2017; Sponheim et al., 2020; Vangroenweghe et al., 2015). Interestingly, lung tissue homogenate samples can yield false negative results or higher Ct values compared to bronchial swabs (Burrough et al., 2018).

Alternative approaches to testing individual specimens have also been investigated. Sponheim et al. (2021) showed that pooling strategies for *M. hyopneumoniae* detection by PCR in tracheal secretions at late stages of infection increased testing affordability while retaining sufficient diagnostic sensitivity. *Mycoplasma hyopneumoniae* has been also detected by PCR in aggregate samples such as oral fluids, where it showed low sensitivity and consistency, precluding its use as a disease surveillance and monitoring tool (Hernandez-Garcia et al., 2017; Pieters et al., 2017), and more recently in processing fluids (Vilalta et al., 2019, 2020). This latter finding challenges the current evidence that the presence of *M. hyopneumoniae* is restricted to the respiratory tract, thus needing further investigation. Finally, *M. hyopneumoniae* has been also detected by PCR in environmental samples such as air, air deposition particles, and farm stall surfaces (Garza-Moreno et al., 2022; Otake et al., 2010).

Other, less frequently utilized methods to detect *M. hyopneumoniae* nucleic acids are DNA *in situ* hybridization (ISH; (Kwon et al., 2002)) and RNAscope (Surendran Nair et al., 2018). Similar to IHC, both methods have the ability of localizing the bacteria in formalin-fixed paraffin embedded tissues.

Although PCR testing for the detection of *M. hyopneumoniae* is the cornerstone for the majority of diagnostic investigations, as well as for surveillance and monitoring purposes, the interpretation of the results generated by this technique can be challenging in certain scenarios. Since DNA molecules are detected, and these remain present in non-viable cells and in the environment for long periods of time, there is no direct relationship between PCR detection and viability of the detected *M. hyopneumoniae* cells (Josephson et al., 1993). Thus, in some scenarios such as in antibiotic-based control strategies or in elimination programs, the detection of *M. hyopneumoniae* by PCR could represent either the presence of viable cells or uncleared remnants of genetic material from non-viable cells. In order to overcome the limitations that the diagnosis of *M. hyopneumoniae* has in certain circumstances, research aiming at determining the viability of bacterial cells is needed.

1.4 Viability determination in bacteria: focus on M. hyopneumoniae

The first questions on the viability of microbes could be as old as the birth of microbiology, when in 1683, Anton van Leeuwenhoek recorded the first observation of bacteria. Two centuries later, Robert Koch defined the pure culture and colony (Koch, 1883), and the first microbiological techniques for the quantitative estimation of the number of viable cells in bacterial samples. Indeed, bacterial culture has been the standard technique for the determination of microbiological viability, since the observable division of a single cell into colonies on agar plates or liquid medium provides evidence that the cells are alive (Bogosian & Bourneuf, 2001). However, there are several characteristics other than cultivability that are considered to define that a bacterium is alive. It is generally accepted that a cell must be intact, capable of reproduction, and metabolically active in order to be considered viable, and different viability assessments are designed to measure one or more of these properties (reviewed by Emerson et al., 2017; S. S. Kumar & Ghosh, 2019). Thus, in general, culture-independent viability determination assays can be classified by the way they address the three main aspects of bacterial viability: the existence of an intact, functional cell membrane, the presence of cellular metabolism or energy, or finally, the presence of self-replicating DNA that can be transcribed into RNA and which can be subsequently translated into protein (Hammes et al., 2011).

1.4.1 *Culture-dependent methods*

Successful culturing is a clear indication that an organism is alive, but unsuccessful culturing is not proof of lack of viability. *Mycoplasma hyopneumoniae* is cultured in Friis-based medium and incubated from days to weeks at 37°C in an aerobic atmosphere (Pieters & Maes, 2019). Bacterial growth is not determined by turbidity, but by acidification of the medium, observed by the change in color of the phenol red indicator. Since the growth of *M. hyopneumoniae* in solid agar medium can take weeks and is very inconsistent (Cook et al., 2016; Garcia-Morante et al., 2018), the colony forming units (CFU) method of bacterial titration is not employed. Instead, the color changing units (CCU; Stemke & Robertson, 1982) is the most common method of titration used. However, this ten-fold dilution approach is an indirect, subjective and low-accuracy method (Calus et al., 2010).

As mentioned above, bacterial isolation of *M. hyopneumoniae* from clinical samples is very fastidious and has very low sensitivity, even when procedures to optimize animal selection, sample collection and handling and laboratory processing have been described

(Anderson et al., 2016). Thus, the development of culture-independent methods to assess *M. hyopneumoniae* viability is needed.

1.4.2 *Culture-independent methods*

1.4.2.1 *Techniques based on membrane integrity*

The outer cell membrane is critical for cell homeostasis, as it defines the individual cell and provides physical compartmentalization with the external environment. Thus, membrane integrity is considered to be a biomarker for viable cells, and it can be measured by the use of selective stains (often called viability dyes) coupled with visualization methods such as microscopy or flow cytometry.

Propidium iodide (PI) is one of the most common fluorescent stains to determine viability by membrane integrity. Propidium iodide is a red-fluorescent dye that does not permeate cells with intact membranes. Thus, if the cell membrane is compromised, PI crosses the membrane and binds to internal nucleic acids (Williams et al., 1998). Because of its fluorescent properties, PI can be detected via epifluorescence microscopy or flow cytometry (Pietkiewicz et al., 2015).

The use of this technique has not been described for *M. hyopneumoniae*. However, the availability of commercial kits (LIVE/DEAD BacLight® Bacterial Viability Kits, ThermoFisher) that use PI in combination with SYTO 9 (a green fluorescent total nucleic acid dye used to stain all cells) could open the door to its use in the future. The minute size of *M. hyopneumoniae* could be anticipated as a possible obstacle for the success of the technique.

Other types of viability dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA) can be used, coupled with a DNA amplification-based method such as PCR, to determine cell membrane integrity. This technique is most commonly called ‘viability PCR’ in the literature (Emerson et al., 2017). In viability PCR, samples are first treated with a viability dye such as EMA, that penetrates damaged cell membranes. Once inside the cells, the nucleic acid intercalating dye binds to DNA, and upon photoactivation, the dyes form covalent bonds resulting in irreversible binding to nucleic acids (Soejima et al., 2007). Then, when DNA from a treated sample is amplified by PCR, only DNA from cells with intact membranes is to be amplified, as the degraded DNA from extracellular DNA and from cells with compromised membranes provide poor templates for DNA amplification. Therefore, EMA-PCR can be used as a tool for selective detection of nucleic acids from live cells (Nocker et al., 2006; Pan & Breidt, 2007).

Anderson et al. (2015) explored the use of a viability PCR via EMA to determine *M. hyopneumoniae* viability *in vitro.* That study showed that the method was not suitable for differentiating live from heat-inactivated cultures, as EMA was taken up and intercalated in all samples, regardless of known viability status and photoactivation settings. The lack of cell wall in the case of *M. hyopneumoniae* was hypothesized to play a role on the observed results.

1.4.2.2 *Techniques based on transcription (RNA-based methods)*

Ribonucleic acid (RNA) can be used as a molecular target for viable microbes, since transcription is one of the first cellular changes in response to stimuli and RNA has much shorter half-life than DNA. Specifically, messenger RNA (mRNA) is extremely short-lived, with an average half-life of minutes in active cells and even less as a free molecule in the environment (Keer & Birch, 2003; Moran et al., 2013). Ribosomal RNA (rRNA), has a half-life of days and is much more abundant in cells (up to 90% of total cellular RNA in bacteria is rRNA; Evguenieva-Hackenberg & Klug, 2011) and so, it has been used as a less accurate viability indicator in low-biomass samples, such as water or soil samples.

The development of reverse-transcriptase PCR assays targeting mRNAs of different bacterial species has been described, and has been applied for the exclusive detection of viable bacterial cells (Coutard et al., 2005; Lleò et al., 2000; Zhou et al., 2014). In *M. hyopneumoniae*, reverse-transcriptase PCR targeting mRNA has been also developed and applied in transcriptional analyses to assess the metabolic responses of bacteria exposed to different types of stimuli, such as heat-shock (Madsen et al., 2006), exposure to hydrogen peroxide (Schafer et al., 2007), iron depletion (Madsen et al., 2006) or during infection in live pigs (Madsen et al., 2008). However, this technique has not been used to detect and discriminate viable from non-viable *M. hyopneumoniae* cells.

Reverse-transcriptase PCR has been, however, used for this purpose in another *Mycoplasma* species of interest in animals, such as *M. synoviae,* a poultry pathogen that causes respiratory disease and decreased egg production of turkeys (Marois et al., 2002, 2005). In those studies, the mRNA coding for a membrane protein and the 16S rRNA of *M. synoviae* were the targets. In *in vitro* experimental conditions, mRNA and rRNA were detected by reverse-transcriptase PCR up to 20 minutes and 23 hours post-inactivation via osmotic shock, respectively.

From a practical point of view, though, working with RNA is more complicated than with DNA. The highly labile nature of RNA can make sample processing a challenge, and losses up to 80% of the total mRNA present in a sample have been reported (Johnson et al., 2005; Kim et al., 2012). Also, obtaining clinical specimens for RNA detection from farm settings could be difficult or perhaps impossible if snap-freezing with liquid nitrogen is deemed required. There have been, however, great advances in the methods for the procurement and storage of samples intended for RNA processing, and the use of RNA stabilization solutions such as RNAlater® and products to prevent RNase contamination of laboratory environments has been standardized (Shabihkhani et al., 2014). Therefore, reverse-transcriptase PCR targeting *M. hyopneumoniae* mRNA could be used for the exclusive detection of viable cells. This method appears to be suitable for both laboratory and field settings if samples collected in the field are stored and transported in a way that the stability of the RNA present in those samples is maintained.

1.4.2.3 *Techniques based on translation (protein-based methods)*

In a living organism, there is transcription from DNA into RNA and then translation from mRNA into new proteins. Protein-based methods of determining bacterial viability and metabolism target the synthesis of new proteins. Translational activity has been measured by the use of a technique called biorthogonal noncanonical amino acid tagging (BONCAT), which is based in the incorporation of artificial amino acids into newly synthesized proteins by metabolically active cells (Hatzenpichler et al., 2014). When the artificial amino acids are designed to have fluorescent properties, highly specific azide-alkyne click chemistry can be used to detect fluorescent polypeptide chains that are visible via fluorescent microscopy or flow cytometry (Steward et al., 2020). This technique has been used by researchers at the University of Minnesota to reveal the translationally active subpopulations of the microbiota of human patients with cystic fibrosis (Valentini et al., 2020) and could potentially be used for *M. hyopneumoniae*, at least *in vitro*.

1.4.2.4 *Techniques based on cellular metabolism*

Adenosine triphosphate (ATP) is an organic compound that provides energy to drive and support many processes in living cells and thus, it can be used as a biomarker for viable organisms. Methods to detect ATP involve the addition of an ATP-releasing reagent to lyse cells and release ATP, which, in the presence of luciferase, produces light. The intensity of the emitted light is then measured as relative light units (RLU), which are interpreted as ATP concentration (Stanley, 1989). ATP luminometry has been used to enumerate and titrate *M. hyopneumoniae* in culture (Calus et al., 2010; Garcia-Morante et al., 2018; Stemke & Robertson, 1990). However, the applicability of this method in clinical specimens is uncertain, as the release of ATP from host cells could be expected to interfere with light production and overestimate ATP concentrations.

1.4.3 *Transmission experiments and bioassays*

Bacterial transmissibility and infectivity have been used as a proxy to determine bacterial viability, as, for a pathogen, being alive is a prerequisite to be infectious. However, not all viable pathogens are transmitted, as transmission depends on several factors such as infectious dose, time and route of exposure, and susceptibility of the recipient host, among others. Additionally, transmission experiments and animal bioassays can be non-practical and expensive, as they require the use of certain numbers of animals in very defined housing conditions. In addition, these methods are usually against the current perspective of the use of animals in science, summarized by the three Rs (replacement, reduction, refinement) (Hubrecht & Carter, 2019). Nonetheless, transmission experiments and bioassays have been and continue to be used in *M. hyopneumoniae* research to infer information of the viability of bacteria in different situations, such as after antibiotic treatment (Betlach et al., 2021) or in collected aerosol samples (Otake et al., 2010).

In conclusion, important advancements in the knowledge of *M. hyopneumoniae* diagnostics have been achieved over the years thanks to the research compiled in this literature review. However, there are still knowledge gaps, some of them derived from imperfect testing capabilities to determine bacterial viability for this pathogen. This dissertation will aim to overcome some of the diagnostic limitations associated with PCR testing and bacterial viability of *M. hyopneumoniae.*

Chapter 2: Longitudinal evaluation of *Mycoplasma hyopneumoniae* detection in processing fluids in swine breed-to-wean farms

2.1 Summary

Mycoplasma hyopneumoniae is one of the main pathogens associated with respiratory clinical disease in pigs worldwide. Even though the tissue tropism for this bacterium is considered to be restricted to the respiratory tract, extra-pulmonary localization has been seldomly described in experimental infection studies. The recent detection by PCR of *M. hyopneumoniae* in processing fluids (PF) in endemically infected sow farms was unprecedented and warranted further research. The objective of this study was to longitudinally investigate the detection of *M. hyopneumoniae* in PF in breeding herds with various sanitary status and management conditions.

Mycoplasma hyopneumoniae was regularly detected by PCR in PF in positive farms, while it was generally not detected in negative farms. An association between bacterial detection in PF and gilt litters was observed, while the same was not true for seasonality. Seven DNA sequences from three farms were nearly identical and exhibited 99.7-100% homology to the *M. hyopneumoniae* J strain.

Although the source of the detected *M. hyopneumoniae* genetic material in PF cannot be unequivocally ascertained, results from this study highly suggest that detection originated from environmental contamination of the samples with vaccine products. The extended stability of DNA molecules in the environment, paired with the high sensitivity of PCR assays, may lead to unexpected findings, making the correct interpretation of molecular diagnosis results challenging. Results from this study highlight the importance of good practices in sample collection, aiming at minimizing pre-analytical error and contributing to the correct interpretation of molecular diagnostics.

2.2 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is an important respiratory pathogen and the causative agent of enzootic pneumonia in pigs (Pieters & Maes, 2019). The bacterium attaches to and perturbs the homeostasis of ciliated epithelial cells in the airways of the respiratory tract, causing ciliostasis and promoting a prolonged inflammatory reaction (Tajima & Yagihashi, 1982). This allows upper respiratory tract bacteria, such as *Pasteurella multocida* and/or *Bordetella bronchiseptica*, to reach the lower respiratory tract causing suppurative bronchopneumonia. Additionally, the detrimental effects of respiratory viruses are also potentiated by *M. hyopneumoniae* infections (Marois-Créhan et al., 2020). Although it is broadly accepted that the presence of *M. hyopneumoniae* is restricted to the respiratory tract, extra-respiratory detection and/or isolation of this bacterium in experimental infection studies has been occasionally described in the literature, in tissues such as in brain (Friis, 1974), liver, kidney, spleen (Le Carrou et al., 2006; Marois et al., 2007; Woolley et al., 2012) and in boar semen (Milovanovi et al., 2017). The detection of *M. hyopneumoniae* in the above-mentioned tissues suggests that there might be systemic circulation to other body systems.

Processing fluids (PF) are an aggregated sample type composed of the serosanguineous transudate obtained from the testicles and tails, at castration and tail docking, respectively, from 3 to 5-day-old piglets, a process known as “processing” in the United States. The collection of this specimen has become a standardized and widely used sampling strategy to evaluate the presence of viral pathogens, such as Porcine Reproductive and Respiratory Syndrome virus, in very young piglets (Vilalta et al., 2018). Other viral pathogens that can be detected in PF are Porcine Circovirus 2 and Senecavirus A. To date, the use of PF for detection of bacterial infectious agents, endemic to sow farms, has not been investigated. In the case of *M. hyopneumoniae*, the presumed lack of multiorgan spreading of the bacterium outside the respiratory system was an obvious reason to disesteem investigation on its detection in PF. However, a preliminary study conducted by our research group in a positive farm described the detection of *M. hyopneumoniae* by PCR in 38% of the litter PF tested (Vilalta et al., 2019). Importantly, results also indicated a possible parity effect on the detection of the bacterium, as the relative risk of detecting *M. hyopneumoniae* in PF from gilt litters was two times higher than in older parities. In addition, daily sampling of pooled PF through December and January in the same farm revealed the detection of the bacterium in 50% of the samples. Although, a seasonal effect could not be ascertained, as sampling was restricted to a two-month period.

In parallel, the detection of *M. hyopneumoniae* in PF in a previously negative sow farm was observed at the same chronological time that a clinical respiratory disease outbreak caused by *M. hyopneumoniae* occurred, stressing the hypothetical value that this clinical specimen could have to detect the infection in breed-to-wean farms (Vilalta et al., 2020).

The unforeseen detection of *M. hyopneumoniae* in PF raised several questions regarding the epidemiology and pathogenesis of this bacterium in pigs, questions that could not be answered with the current knowledge of this disease entity. A further comprehensive investigation aiming at expanding the information and overcoming the limitations of the aforementioned findings was the aim of the present study. Specifically, the association between the detection of *M. hyopneumoniae* in PF and the farm status for this bacterium, season and dam parity was longitudinally investigated.

2.3 Materials and Methods

2.3.1 *Study farms*

This investigation was performed as a longitudinal study in commercial sow farms in the U.S. Ten sow farms (1-10) from three production systems (A-C) were enrolled in the study at different times. Farms were classified as either positive (n=5) or negative (n=5) based on their *M. hyopneumoniae* infection status, according to Garza-Moreno et al. (2018). One of the enrolled positive farms went through a depopulation-repopulation process while participating in the study.

Inclusion criteria were applied in order to enroll the farms in the study. Briefly, the criteria included having a well-established *M. hyopneumoniae* status based on auditable diagnostic testing, willingness to disclose farm management and health-related information and capability of collecting and submitting samples monthly for an entire year. Exclusion criteria included a non-justified failure to collect three consecutive monthly samples. Additionally, a one-page sampling protocol and a schematic flow diagram detailing sample collection and emphasizing the need to change gloves between litters was prepared, both in English and Spanish. The sampling protocol was provided to farm personnel along with sampling materials and PPE. Sampling materials were pre-labeled in our research laboratory to facilitate as much as possible the sample collection by farm staff.

2.3.2 *Sample collection*

Thirty individual PF samples, ten each from gilt, parity two and parity three and older litters, were collected monthly by farm staff at each farm following regular farm procedures. The samples consisted of one plastic bag (Whirl-pak®, Madison, WI) containing all testicles from a single litter after processing. Tails were not included in the sample. Each bag was labeled with the litter ID, dam parity, and date of sample collection. All bags collected in the same month were frozen (-20°C) at the farm prior to being mailed to the laboratory. At arrival to the laboratory, samples were thawed at room temperature, the fluid obtained from the testicles was collected using individually wrapped Pasteur pipettes, and 1.5 mL were aliquoted into a microtube in duplicate. Testicles were discarded after the liquid was collected.

2.3.3 *Sample processing*

Individually aliquoted PF were pooled by five, maintaining the same parity and month within every pool. Pooled samples were submitted to the University of Minnesota Veterinary Diagnostic Laboratory (UMN-VDL) and processed for nucleic acid extraction using MagMAX CORE Nucleic Acid Purification Kit (ThermoFisher Scientific, Waltham, MA, USA). The purified genetic material was tested by PCR using the IDEXX RealPCR™ *M. hyopneumoniae* DNA Mix and reagents of the IDEXX RealPCR platform following the manufacturer's instructions (IDEXX Laboratories Inc, Westbrook, ME, USA). Samples with a Ct value <40 were considered positive. In addition, 14 pooled samples with the lowest Ct values, representing five farms of two production systems, were split up and the samples making up the pool were individually tested. The individual samples with lowest Ct values were subject to P146 adhesion-like gene DNA sequencing per established protocol at the UMN-VDL. The P146 gene contains three variable repeat regions and two variable coiled-coil regions (Felde et al., 2018). Since each P146 nucleotide sequence varies in length due to the presence of multiple repeat regions, the nucleotide sequence was translated to amino acids for P146 phylogenetic analysis. An amino acid percent identity matrix, containing the PF sequences and reference sequences obtained from GenBank, was constructed. The reference sequences included the following strains: 232 (USA, GenBank: AE017332.1; Minion et al., 2004), 7448 (Brazil, GenBank: AE017244.1; Vasconcelos et al., 2005), J (UK, GenBank: AE017243.1; Vasconcelos et al., 2005), 168 (China, GenBank: CP002274.1; (Liu et al., 2013), 168-L (China, GenBank: CP003131.1; Liu et al., 2013), F7.2C (Belgium, GenBank: DQ088147.1; Stakenborg et al., 2006), and BQ14 (France, GenBank: AF279908.1).

2.3.4 *Data analysis*

Results were statistically analyzed by constructing a mixed effects logistic regression model with the PCR result (positive/negative) as the dependent variable and the parity group, season and *M. hyopneumoniae* farm status as predictors**.** Sequencing results were analyzed using the Mega X software (S. Kumar et al., 2018).

2.4 Results

A total of 2,923 individual litter-PF were received and aliquoted. After pooling, a total of 602 samples were tested by PCR and 263 were positive (44%). The Ct values of the positive samples ranged from 25.03 to 39.94. The PCR positivity rate of *M. hyopneumoniae* in PF by farm status, month of the year and sow parity is shown in Table 2.1. The positivity rate was higher than 90% in positive farms and between 5-7% in negative farms. There was a statistically significant association between *M. hyopneumoniae* detection in PF, farm status (adjusted odds ratio 8,629.3; 95% CI: 146.7 – 507,598.7; p-value <0.001) and winter season (adjusted odds ratio 9; 95% CI: 2.7 - 29.4; p-value <0.001). An association between PCR detection and parity was not observed (Table 2.2).

A selection of 14 pooled samples, representing various positive farms from different production systems and months of collection were split and the samples within the pool were individually tested. From the 14 individual PF samples that were subject to P146 DNA sequencing, one complete and six partial sequences were obtained. For the rest of the individual samples, sequencing was not successful despite moderately low Ct values (Table 2.3). Sequences represented three farms from one production system. In the amino acid percent identity matrix, constructed with the farm sequences and reference sequences obtained from GenBank, the seven farm sequences were identical or almost identical (maximum percentage of amino acid substitution of 0.19%), and highly homologous (99.7-100% amino acid homology) to the *M. hyopneumoniae* J strain (Table 2.4).

2.5 Discussion and Conclusions

Mycoplasma hyopneumoniae was frequently detected by PCR in PF from positive farms, both in terms of quantity of genetic material and consistency of detection throughout time, while it was generally not detected in negative farms. Thus, it seems evident that the detection of *M. hyopneumoniae* genetic material by PCR in PF might be a common finding rather than a sporadic event, as the detection was consistent across various farms of different production systems, regardless of sow parity.

The detection of the bacterium in processing fluids was a remarkable finding, since *M. hyopneumoniae* is considered to be restricted to the respiratory tract. Nonetheless, numerous scientific publications describe the extra respiratory detection and/or isolation of *M. hyopneumoniae,* although the data has been obtained from a limited number of pigs that have been inoculated through the respiratory route under experimental conditions. Thus, it seems plausible that *M. hyopneumoniae* cells are mechanistically capable of disseminating systemically, at least transiently and for some particular strains (Woolley et al., 2012). In addition to experimental *in vivo* studies, several *in vitro* studies have suggested ways in which *M. hyopneumoniae* cells could traffic outside the respiratory tract. One study in particular showed that *M. hyopneumoniae,* a bacterium that is considered an extracellular pathogen, had the ability to interact with the surface of cultured porcine epithelial cells, entered the cytoplasm, survived phagolysosomal fusion and resided intracellularly within endosomes (Raymond et al., 2018). Another *in vitro* study using an air-liquid interface culture system for porcine bronchial epithelial cells, described the ability of *M. hyopneumoniae* to disrupt the epithelial barrier and migrate to the basolateral chamber through the paracellular route (Wang et al., 2020). In both studies, the authors suggested that these might be mechanisms by which *M. hyopneumoniae* could disseminate extrapulmonary, evading the immune system and creating persistent infections and reinfections in pigs. Nevertheless, the mechanisms by which *M. hyopneumoniae* can reach the testicles and be detected in PF collected from one to three-day-old piglets are unclear. First, vertical transmission of *M. hyopneumoniae* has not been described and thus, it is unknown when piglets would eventually be exposed to or colonized by the bacterium, either being *in utero*, at birth during passage through the birth canal, or horizontally from the dam very early after birth. In addition, in the hypothetical case that piglets would become colonized, it is questionable that the bacterium would gain access to the testicles, an immune-privileged organ, due to the presence of the blood-testis barrier (Mruk & Cheng, 2015). However, it could still be present in the transudate from the blood vessels and lymphatics of the peritesticular tissues.

Considering that the detection of *M. hyopneumoniae* in PF was very consistent throughout time and high relative bacterial loads were observed, efforts at characterizing the nature and possible origin of the detected genetic material followed, and DNA sequencing of a selection of samples was performed. An extremely high P146 DNA sequence homology was observed among the *M. hyopneumoniae* detected in different farms and compared to the J strain, which has been historically used for vaccine production and is used in commercial bacterin products (Garza-Moreno et al., 2018). Thus, it can be hypothesized that the origin of the detected genetic material may be associated to environmental contamination of the PF with *M. hyopneumoniae* vaccine products rather than true presence of the bacterium in the testicles or in the associated serosanguineous transudate. This is consistent with the predominant detection in positive farms, as the inclusion of *M. hyopneumoniae* in the vaccination protocol for weaning pigs usually occurs when the sow farm and the downstream flow is positive, while it may not occur when the sow farm is negative. Additionally, there was no association between PCR detection and parity in this study, while infection with *M. hyopneumoniae* is known to be more prevalent in gilts than older parity sows (Vangroenweghe & Thas, 2021).

Nevertheless, a positive PCR result for *M. hyopneumoniae* may represent the presence of more than one bacterial strain within a single sample. Sequencing data obtained from the PF samples of this study could suggest that the detected genetic material most likely originated from a commercial vaccine product, as there was nearly identical homology of the sequenced samples to the J strain of the bacterium. However, generally during the sequencing process, only the sequence of the strain that is present in greater copy numbers may be detected. This type of amplification bias may have occurred in the present study, in the way of preferential amplification towards the vaccine strain. Therefore, with the data obtained from this study, there is not enough evidence to preclude the presence of wild type *M. hyopneumoniae* in the PF evaluated by PCR. DNA sequencing was not successful in half of the samples in which the technique was attempted, and partial sequences were obtained in most successful attempts, even if the Ct values were considered moderately low. It could be hypothesized that the method of bacterin inactivation in the vaccine preparation, as treatment with formaldehyde may cause fragmentation and coiling of DNA, may have contributed to an acceptable template for PCR testing but a poor template for sequencing.

In previous studies on the detection of *M. hyopneumoniae* in PF, authors collected samples aiming at minimizing environmental contamination by cleaning the incision area in the scrotum with a disinfecting wipe or wearing new gloves between sampled litters. However, the authors acknowledged that the possibility of external contamination of the PF could not be ruled out. In fact, it was recently shown that *M. hyopneumoniae* DNA can be detected in the air, stall surfaces and the dam skin and mucosae in positive farms (Garza-Moreno et al., 2022). In the present study, even if the sampling protocol indicated that personnel was required to change gloves between sampled litters, and gloves were provided in order to promote application of the protocol, sample collection was not supervised by researchers and thus, the environmental contamination of samples cannot be discarded. Also, the majority of the positive farms belonged to the same production system, and the obtained sequencing data originated from different farms of the same production system. Thus, further efforts aiming at expanding data collection, including additional farms from other production systems, should be considered.

The prolonged stability of DNA in the environment, together with the high sensitivity of the PCR assays, may lead to unexpected molecular diagnostic test results, in which case the origin of the detected genetic material needs to be traced down. In the present study, the detection of *M. hyopneumoniae* in PF may have originated, at least in part, from contamination from inactivated vaccine. This source of environmental genetic material is not to be ignored, as recent studies reinforced the importance that this issue can have on diagnostic results interpretation. Hensch (2021) and collaborators showed that post-vaccination environmental contamination of samples was possible even with the collection of tracheal secretion samples, the preferred sample type for *ante mortem* detection of *M. hyopneumoniae.* Additionally, Weidmayer (2020) and collaborators described the prolonged persistence of detection of DNA in the environment, showing that commercial bacterin DNA could be detected for up to 28 days after intentional deposition on farrowing stalls and heating mats under field conditions. In the case of contamination of PF with vaccine products, one other hypothesis is that personnel performing castration of piglets may have been previously vaccinating weaning pigs, and thus, there is the potential for cross-contamination of the PF with the worker's hands and/or clothes.

The diagnostic challenges of this and other studies describing environmental contamination of samples emphasize that, for an appropriate interpretation of *M. hyopneumoniae* PCR results, efforts should be made to minimizing the possibility of pre-analytical error, that is, all the procedures that occur before the sample is analyzed, including sample collection, handling and transport (Plebani, 2012). Determining clean and dirty areas when sampling, as well as cleaning and disinfecting sampling materials regularly would be beneficial in most cases and for most pathogens. These measures should be maximized in high-risk scenarios, such as in the monitoring of gilts for introduction into a naïve herd, especially when gilts have been vaccinated prior to sampling at the gilt development unit.

Bacterial isolation is seldomly performed as a diagnostic technique to evaluate the presence of *M. hyopneumoniae* in a sample due to its low sensitivity (Chae et al., 2021; Garcia-Morante et al., 2022). Hence, PCR testing is the most used assay for this matter and exhibits very high sensitivity, specificity, and rapid turnaround time. However, the assay is not able to determine the viability of the detected genetic material and thus, it might lead to interpretation issues. In the present study, it was not possible to determine if the detected genetic material was sourced by an inactivated bacterin or by a viable *M. hyopneumoniae* cell. Having the ability to determine the viability of the detected *M. hyopneumoniae* is essential when evaluating and monitoring the efficacy of control measures. Additionally, further research to investigate the characteristics of the persistence of detection of DNA from non-viable *M. hyopneumoniae,* both *in vitro* and *in vivo,* should be persued.

In summary, nucleic acid detection of *M. hyopneumoniae* by PCR testing is and will continue to be a commonly used diagnostic technique based on its high detection accuracy. Nevertheless, care should be taken when collecting, handling, and transporting samples to the laboratories, always aiming at minimizing both cross- and environmental contamination. Also, since *M. hyopneumoniae* PCR detection in clinical samples is based on targeting bacterial DNA and current assays do not differentiate between viable and non-viable bacteria, further research on developing culture-independent methods to assess bacterial viability for this pathogen is warranted.

Table 2.1 *Mycoplasma hyopneumoniae* processing fluids PCR positivity rate by farm status, month of the year and sow parity.

	Negative farm status			Positive farm status			
Month/Parity	Gilt	P2	P3+	Gilt	P2	P3+	**Total**
January	17 (2/12)	17 (2/12)	25 (3/12)	100 (7/7)	100 (8/8)	100 (7/7)	**50 (29/58)**
February	8 (1/12)	10 (1/10)	7 (1/13)	100 (7/7)	100 (7/7)	100 (8/8)	**44 (25/57)**
March	10 (1/10)	0 (0/10)	0 (0/10)	100 (7/7)	100 (7/7)	100 (8/8)	**44 (23/52)**
April	8 (1/12)	7 (1/13)	0 (0/11)	100 (6/6)	100 (6/6)	100 (6/6)	**37 (20/54)**
May	0 (0/8)	0 (0/8)	0 (0/8)	100 (6/6)	100 (6/6)	100 (6/6)	**45 (19/42)**
June	0 (0/6)	0 (0/6)	0 (0/6)	86 (6/7)	83 (5/6)	80 (4/5)	**42 (15/36)**
July	0 (0/10)	0 (0/10)	0 (0/10)	67 (4/6)	83 (5/6)	100 (6/6)	**31 (15/48)**
August	20 (2/10)	20 (2/10)	20 (2/10)	86 (6/7)	71 (5/7)	88 (7/8)	**46 (24/52)**
September	0 (0/8)	0 (0/8)	0 (0/8)	90 (9/10)	100 (10/10)	82 (9/11)	**51 (28/55)**
October	0 (0/8)	0 (0/8)	0 (0/8)	88 (7/8)	100 (9/9)	100 (10/10)	**51 (26/51)**
November	10 (1/10)	0 (0/10)	0 (0/10)	86 (6/7)	100 (7/7)	100 (6/6)	**40 (20/50)**
December	0 (0/8)	0 (0/8)	0 (0/8)	88 (7/8)	86 (6/7)	75 (6/8)	**40 (19/47)**
Total	**7 (8/114)**	**5 (6/113)**	**6 (7/114)**	**91 (78/86)**	**94 (81/86)**	**92 (83/89)**	**44 (263/602)**

Values represent percentage of positive samples (positive/total)

Table 2.2 Mixed effects logistic regression analysis of the *Mycoplasma hyopneumoniae* detection in processing fluids by PCR with parity, season and farm status as dependent variables.

Variable	Levels	OR (95% CI)	p-value
Parity	P3+	1.1 (0.5 - 2.5)	0.89
	P2	1.4 (0.6 - 3.3)	0.45
	P1	Reference	
Season	Spring	1.9 (0.7 - 5.4)	0.23
	Summer	1.1 (0.4 - 3.5)	0.84
	Winter	9 (2.7 - 29.4)	<0.001
	Fall	Reference	
Farm status	Positive	8629.3 (146.7 - 507598.7)	<0.001
	Negative	Reference	

Table 2.3. Selection of pooled samples individually tested and subject to *Mycoplasma hyopneumoniae* DNA sequencing.

					Individual PCR Ct values					
Farm	Production company	Month	Parity	Pool PCR Ct value	1	2	3	4	5	Sequencing
4	A	September	P2	29.52	**31.12**	32.05	-	-	-	Insufficient nucleic acid for sequencing
7	C	August	P3+	27.35	**25.44**	32.49	33.38	31.11	35.63	Complete sequence
7	C	September	P2	29.23	**27.55**	33.7	28.2	30.6	29.79	Partial sequence
7	C	October	P2	28.37	**32.92**	34.24	34.39	33.12	34.69	Insufficient nucleic acid for sequencing
7	C	November	P2	25.03	**22.5**	29.39	28.11	32.3	26.5	Insufficient nucleic acid for sequencing
7	C	December	P2	27.1	**24.29**	29.59	27.11	26.87	26.04	Partial sequence
7	C	January	Gilt	25.78	**24.55**	29.34	24.63	26.02	25.3	Partial sequence
7	C	February	P2	27.76	**26.53**	31.01	29.1	34.98	31.09	Insufficient nucleic acid for sequencing
7	C	March	P2	29.33	**28.5**	30.6	29.51	30.34	32.99	Insufficient nucleic acid for sequencing
8	C	September	Gilt	31.86	**29.84**	35.42	32.13	31.15	n/a	Partial sequence
9	C	September	P2	27.25	**25.31**	30.63	26.79	27.6	n/a	Partial sequence
9	C	January	Gilt	27.24	**26.03**	28.06	30.13	26.12	30	Insufficient nucleic acid for sequencing
9	C	February	Gilt	26.98	**26.3**	31.27	27.47	28.16	31.34	Partial sequence
10	C	August	P3+	31.31	**27.88**	32.37	31.31	34.24	30.08	Insufficient nucleic acid for sequencing

A dash indicates that the sample was negative. Non-applicable (n/a) indicates that sample was not available (pool was composed of four individual samples instead of five). The individual samples with the lowest Ct value within a pool are highlighted in bold, as they were selected for DNA sequencing.

Table 2.4 Amino acid percent identity matrix containing the processing fluid sequences and *Mycoplasma hyopneumoniae* reference sequences.

	Processing fluid sequences							*M. hyopneumoniae* reference strains						
	Farm 7 August	Farm 7 September	Farm 7 December	Farm 7 January	Farm 8 September	Farm 9 September	Farm 9 January	232	7448	J	168L	168	F7.2C	BQ14
Farm 7 August														
Farm 7 September	0.00													
Farm 7 December	0.00	0.00												
Farm 7 January	0.30	0.00	0.00											
Farm 8 September	0.00	0.00	0.00	0.00										
Farm 9 September	0.13	0.00	0.00	0.13	0.00									
Farm 9 January	0.19	0.00	0.00	0.19	0.00	0.00								
232	1.72	2.50	2.75	1.92	2.23	2.14	1.94							
7448	2.73	3.70	4.80	2.99	3.40	3.44	3.60	2.88						
J	0.00	0.00	0.00	0.30	0.00	0.13	0.20	1.66	2.66					
168L	3.13	4.94	3.67	3.74	5.80	4.16	2.83	2.88	3.33	3.06				
168	3.13	4.94	3.67	3.74	5.80	4.16	2.83	2.88	3.33	3.06	0.00			
F7.2C	4.01	6.79	5.62	5.00	7.93	5.99	4.59	3.83	3.94	3.54	2.82	2.82		
BQ14	2.86	2.35	1.63	2.56	2.88	2.87	2.59	3.11	3.16	2.64	3.96	3.96	4.37	

Chapter 3: Characterizing the detection of inactivated *Mycoplasma hyopneumoniae* DNA in the respiratory tract of pigs

3.1 Summary

A positive *Mycoplasma hyopneumoniae* PCR result in a clinical specimen may represent the mere detection of non-viable bacteria, complicating the diagnostic interpretation. Thus, the objective of this study was to evaluate the PCR detection of non-viable *M. hyopneumoniae* and its residual cell-free DNA in live pigs. Pigs were inoculated with either active or inactivated *M. hyopneumoniae* and were sampled for up to14 days. *Mycoplasma hyopneumoniae* was not detected by PCR at any timepoint in pigs inoculated with the inactivated bacterium, suggesting that in healthy pigs, the non-viable *M. hyopneumoniae* DNA was rapidly sensed and cleared.

3.2 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*), a respiratory bacterial pathogen that attaches to and disturbs ciliated epithelial cells of the airways, causes a highly prevalent disease in pigs named enzootic pneumonia (Pieters & Maes, 2019). Due to its intrinsic fastidiousness to grow in culture, PCR is the assay of choice to detect the presence of this pathogen in a specimen. Thus, a positive PCR result tends to be interpreted as an indication of active infection with *M. hyopneumoniae*. However, one of the most important disadvantages of this diagnostic approach is that PCR can detect nucleic acids from non-viable bacteria (Josephson et al., 1993), potentially misleading the interpretation.

Animal taxonomy and evolutionary biology have been revolutionized by the analysis and characterization of DNA fragments from archeological bones tens of thousands and even millions of years old (Callaway, 2021). Thus, there is no doubt that DNA is a robust and persistent molecule *ex-vivo*, even when subjected to long periods of unfavorable environmental conditions.

The persistence of nucleic acid detection from non-viable cells *in vivo*, although less studied, has been also reported. For instance, microbial cell-free DNA (cfDNA), a highly fragmented nucleic acid released from decomposing cells, can be identified by PCR in specific organs and in the blood (Blauwkamp et al., 2019). Specifically, cfDNA from respiratory pathogens has been detected in cystic fibrosis (CF) human patients (Barrett et al., 2020). In CF, only a subset of the bacterial communities trapped in the mucus are viable and metabolically active and thus, DNA-based detection methods such as PCR are not suitable to track changes in microbiota composition and metabolism (Valentini et al., 2020; Whelan et al., 2017). Similarly, it has been shown that bacterial DNA on the skin surface overrepresents the viable skin microbiome (Acosta et al., 2023). In veterinary medicine, the detection of RNA from a bluetongue virus inactivated vaccine product in the blood of sheep up to nine days after injection has been described using real-time RT-PCR (Steinrigl et al., 2010), as well as in the blood and spleen of cattle (De Leeuw et al., 2015). These findings emphasize the diagnostic challenge that the PCR detection of non-viable pathogens can pose, especially in field applications, such as in the evaluation of antibiotic treatment efficacy or in the determination of bacterial clearance post-infection.

It is currently unknown if, and for how long, the DNA from non-viable *M. hyopneumoniae* can be detectable by PCR *in vivo*, in the respiratory airways of pigs.

Therefore, the objective of this study was to assess the PCR detection dynamics of non-viable *M. hyopneumoniae* DNA in experimentally inoculated pigs.

3.3 Materials and Methods

This study was conducted according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Sixteen pigs from a *M. hyopneumoniae* and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) negative farrow-to-finish farm were randomly selected, at four weeks of age, and transported to the veterinary isolation facilities at the University of Minnesota. Negative status of the source farm was based on historical diagnostic results regarding *M. hyopneumoniae* seroconversion and detection by PCR, as well as lack of disease-associated clinical signs. Pigs were confirmed negative to *M. hyopneumoniae* via species-specific real-time PCR (Strait et al., 2008) and ELISA testing (Idexx, Westbrook, Maine, USA) prior to experimental inoculation.

A lung homogenate containing $1 \times 10^{5.5}$ color changing units/mL of *M. hyopneumoniae* strain 232 (purchased from Iowa State University, Ames, IA, USA) was combined with Friis medium and used as the inoculum for this study, which was administered in either an active or an inactivated form. The active inoculum, containing viable bacteria, was maintained at -80ºC and prepared just minutes prior to inoculation. An inactivated inoculum, containing non-viable *M. hyopneumoniae*, was prepared by autoclaving at 121ºC and 15psi for 40 min, and stored at -20ºC until use. Both, the active and inactivated inocula were tested by PCR prior to use and showed a Ct value of 28.47 and 29.00, respectively. *Mycoplasma hyopneumoniae*-specific bacterial culture was performed on the inactivated inoculum to determine the efficacy of the heat and pressure treatment on viability. Culture conditions consisted of incubation of original and diluted material in liquid medium (ML, Mycoplasma Experience LTD, Surrey, UK) at 37 ºC in agitation (100 rpm) for at least seven days. Growth of *M. hyopneumoniae* was not obtained from the autoclaved inoculum.

The experimental design of this study is depicted in Figure 3.1. All pigs in this study were three-week old females. At time 0, eight pigs were intratracheally inoculated (Gomes-Neto et al., 2014) with 10mL of the inactivated *M. hyopneumoniae* and transferred to a different experimental room. Likewise, eight pigs were inoculated with a similar amount of the active *M. hyopneumoniae* and were transferred to a different experimental room. At 6 and 12 hours post-inoculation (hpi), and at 1, 2, 3, 5, 10, and 14 days post-inoculation (dpi), tracheal secretions (*ante-mortem* samples) were obtained from each live pig, as described by Fablet et al. (2010). A different set of sterile materials and surgical gloves were used for each individual pig at each sampling event. Researchers collecting samples showered in, and changed clothes and PPE prior to entering the two different experimental rooms. Additionally, one pig per group was humanely euthanized at each timepoint and a necropsy was performed. Samples obtained *post-mortem* included tracheal mucosa (obtained via cell scraping), broncho-alveolar lavage fluid (BALF), bronchial secretions (obtained via swabbing), tracheobronchial and mediastinal lymph nodes and spleen. All samples intended to perform PCR testing were frozen at -20ºC until processing. A portion of the right apical lung lobe of each pig was collected and fixed in 10% buffered formalin. Lungs were macroscopically assessed at necropsy and lesions morphologically compatible with pulmonary consolidation were scored based on the percentage of lobe area affected, as described by Straw et al. (1986). Histopathologic analysis was also performed, and lesions indicative of mycoplasmal pneumonia were scored from 0 to 4, per the scoring system described by Woolley et al. (2012). Both macroscopic and microscopic lesion scorings were performed by the same board-certified pathologist while pig identification was masked. Nucleic acids were extracted by using the MagMAX™ CORE Nucleic Acid Purification Kit coupled with MagMAX^{TM} Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). Real-time PCR was performed using the VetMAX™-Plus qPCR Master Mix and VetMAX™ *M. hyopneumoniae* Reagents Kit (Life Technologies, Grand Island, NY, USA), according to the manufacturer's instructions.

3.4 Results

Detection of *M. hyopneumoniae* DNA by PCR was not obtained at any timepoint in pigs inoculated with inactivated bacteria, either in samples collected *ante-mortem* (Table 3.1) or *post-mortem* (Figure 3.2). In pigs inoculated with the active inoculum, *M. hyopneumoniae* was detected by PCR starting at 2 dpi in tracheal secretions and BALF, and at 3 dpi in bronchial secretions, tracheal scrapings and thoracic lymph nodes. PCR detection in pigs inoculated with viable *M. hyopneumoniae* was observed until the final collection timepoint, at 14 dpi (Table 3.1 and Figure 3.2). *Mycoplasma hyopneumoniae* PCR detection was not obtained from the spleen of any of the pigs in the study. Neither macroscopic nor histologic lung lesions compatible with *M. hyopneumoniae* infection were observed in pigs receiving the inactivated inoculum. Cranioventral pulmonary consolidation and *M. hyopneumoniae*-characteristic lesions were macroscopically and histologically observed at 10 and 14 dpi in pigs inoculated with the active bacterium (Table 3.2). Pigs inoculated with either inactivated or viable *M. hyopneumoniae* exhibited histologic lesions consistent with diffuse lymphohistiocytic interstitial pneumonia with arteritis/periarteritis from 6 hpi to 3 dpi.

3.5 Discussion and Conclusions

This study evaluated the PCR detection dynamics of non-viable *M. hyopneumoniae* cells and their residual nucleic acids in the respiratory system of live pigs. Based on the lack of direct relationship between the viability of bacteria and their detection by PCR, it is conceptually possible that a positive PCR result in a clinical specimen represents the mere detection of residual DNA from non-viable microorganisms. In the case of *M. hyopneumoniae*, since culture-dependent methods to assess bacterial viability are impractical, PCR has become the diagnostic assay of choice. Nevertheless, result interpretation can be challenging in certain epidemiologic scenarios. For example, in pigs at chronic stages of disease that have overcome clinical presentation, the detection of *M. hyopneumoniae* by PCR could indicate the presence of viable bacteria that have not yet been cleared from the respiratory tract and may potentially be infectious to others. Alternatively, *M. hyopneumoniae* detection by PCR may represent the presence of DNA remnants of non-viable bacteria that have not yet been cleared. Thus, positive pigs would no longer be truly colonized with viable *M. hyopneumoniae* and would not represent a risk of infection to other pigs.

Results obtained in this study suggest that, in pigs with non-compromised mucociliary apparatuses and immune systems, the DNA of non-viable *M. hyopneumoniae* was not detectable by PCR in the respiratory or the lymphatic systems at all timepoints post intratracheal inoculation. On the contrary, in pigs inoculated with viable *M. hyopneumoniae*, DNA was consistently detected by PCR in different parts of the respiratory system starting at days two or three post-inoculation.

Rapid recognition of an invading pathogen by a host is the first step to create a protective immune response. Different components of the innate immune system cooperate to avoid or minimize the establishment of infectious agents and the development of their detrimental effects. The mucociliary system is one of the first defense mechanisms for invading respiratory pathogens, which become trapped within the mucus layer and are expelled out of the airways by the synchronized movement of cilia. The function of the mucociliary system can be compromised when pigs are exposed to respiratory bacterial infections, most notably *M. hyopneumoniae*, toxic gases such as ammonia, or dust (Maes et al., 2018). Pigs in the present study harbored intact mucociliary clearance apparatuses, potentially minimizing the contact between *M. hyopneumoniae* DNA and the mucosa of the airways and propelling it out of the respiratory system. Other key components of the innate immune response are the different families of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), that are capable of recognizing foreign DNA, particularly TLR9 (Pandey et al., 2014; Uenishi & Shinkai, 2009). TLR2 and TLR6 have been also described to be involved in the recognition of *M. hyopneumoniae* by porcine alveolar macrophages (Muneta et al., 2003). Thus, the presence of DNA and cellular debris from degenerated *M. hyopneumoniae* may have been detected by resident cells of the respiratory innate immune system, such as dendritic cells and macrophages, and cleared. Additionally, both the active and inactivated inocula used in this experiment contained Friis medium, which is supplemented, among other components, with yeast extract and both equine and swine serum, substances that are most likely immunogenic when introduced to the respiratory tract of pigs, exacerbating the response of the immune system. A likely immunogenic response was evidenced by the presence of lymphohistiocytic interstitial pneumonia with arteritis/periarteritis in the lungs of pigs exposed to either type of inoculum (i.e., active or inactivated) within the first three days. Altogether, these mechanisms most likely contributed to non-detectability of the DNA of non-viable *M. hyopneumoniae*. However, pigs housed in commercial farms are commonly exposed to environmental conditions and bacterial infections that weaken the mucociliary apparatus, as well as viral pathogens, such as PRRSV or porcine circovirus, which are known to have an immunomodulatory role, delaying host immune responses (Chand et al., 2012). Thus, the persistence of genetic material from non-viable *M. hyopneumoniae* in pigs housed in farm conditions and subject to coinfections with other pathogens may be extended and hence, further research is warranted.

Interestingly, there was a lack of detection of the bacterium by PCR in the first two days post-inoculation, even in samples from the lower respiratory tract and lymphoid system collected *post-mortem* in pigs exposed to the live bacterium. Similar results were previously reported in nasal and laryngeal secretions and tracheo-bronchial lavage fluid at 2 dpi, and becoming positive at 5 dpi (Pieters et al., 2017). The cause of the transient lack of detectability of live *M. hyopneumoniae* early post-inoculation, as well as the location of *M. hyopneumoniae* within the respiratory system during that time is unknown, and the significance of this finding is, at this moment, uncertain.

In summary, results from this investigation suggested that only viable *M. hyopneumoniae* can be detected by PCR in the respiratory tract of otherwise healthy pigs two days post-inoculation. Ideally, a culture-independent molecular diagnostic method, such as one detecting exclusively viable *M. hyopneumoniae* can aid in investigating the potential persistence of non-viable bacterial cells in the respiratory system. Such viability assay could be applied in situations where treatments or management practices are expected to affect the viability of *M. hyopneumoniae in vivo*.

Table 3.1 Ante-mortem detection of *Mycoplasma hyopneumoniae* by PCR in tracheal secretion samples.

	Time post-inoculation (h = hours; d = days)							
	6 h	12 h	1 d	2 d	3 d	5 d	10 d	14 d
Inactivated inoculum	-							
	-	-						
	-	-	-					
	-	-	-	-				
	-	-	-	-	-			
	-	-	-	-	-	-		
	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-
Active inoculum	-							
	-	-						
	-	-	-					
	-	-	-	38.34				
	-	-	-	33.18	35.1			
	-	-	-	35.2	30.47	33.75		
	-	-	-	37.13	30.96	28.98	26.42	
	-	-	-	-	38.53	30.56	27.92	27.73
Mean Ct ± Standard deviation	-	-	-	36.77 ± 2.26	33.77 ± 3.8	31.09 ± 2.43	27.17 ± 1.06	27.73

Each row represents an individual pig. A dash represents target not detected, while numbers represent Ct values. Gray shaded cells represent samples not collected (pigs euthanized at a previous timepoint).

Table 3.2 Macroscopic percentage of lung with pulmonary consolidation and histopathologic severity of *Mycoplasma hyopneumoniae*-characteristic lesions.

		Time post-inoculation							
		6 h	12 h	1 d	2 d	3 d	5 d	10 d	14 d
Inactivated inoculum	Macroscopic score (%)	0	0	0	1	0	0	0	0
	Histopathologic score (0-4)	0	0	0	0	0	0	0	0
Active Inoculum	Macroscopic score (%)	0	0	0	0	0	0	32	16
	Histopathologic score (0-4)	0	0	0	0	0	0	2	3

Lung lesion scores for pigs euthanized at different times post-inoculation. One pig from each experimental group, inoculated with either the inactivated or the active inoculum, was euthanized at each timepoint and a *post-mortem* lung evaluation was performed. h= hours. d= days.

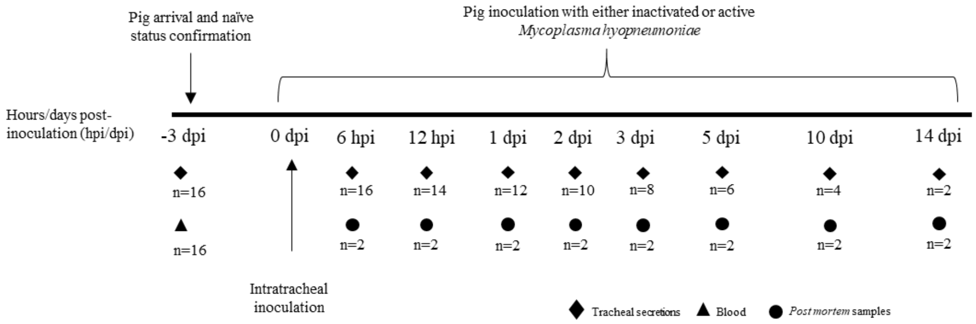

Figure 3.1 Experimental design and sample collection scheme.

Eight pigs were inoculated with inactivated *Mycoplasma hyopneumoniae*, while eight pigs were inoculated with the viable bacterium. Two pigs were humanely euthanized at every timepoint (one pig per experimental group). Black diamonds indicate *ante-mortem* tracheal secretions sample collection. Black triangle indicates blood sample collection. Black circles indicate *post-mortem* sample collection at necropsy, including tracheal scrapings, bronchial secretions, bronchoalveolar lavage fluid, thoracic lymph nodes and spleen. Macroscopic and microscopic lung lesion assessment and grading was performed in all euthanized pigs.

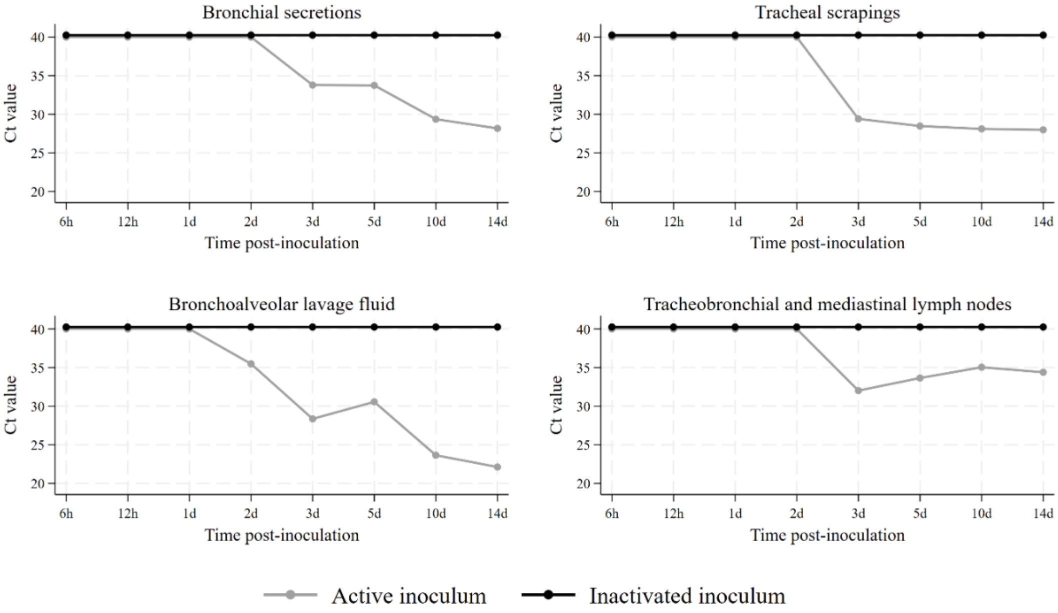

Figure 3.2 *Post-mortem* detection of *Mycoplasma hyopneumoniae* by PCR in various sample types.

Results are expressed as Ct values. Gray dots: Active inoculum. Black dots: Inactivated inoculum.

Chapter 4: Detection of *Mycoplasma hyopneumoniae* viability using a PCR-based assay

4.1 Summary

Mycoplasma hyopneumoniae detection in clinical specimens is accomplished by PCR targeting bacterial DNA. However, the high stability of DNA and the lack of relationship between bacterial viability and DNA detection by PCR can lead to diagnostic interpretation issues. Bacterial messenger RNA is rapidly degraded after cell death, and consequently, assays targeting mRNA detection can be used for the exclusive detection of viable bacterial cells. Therefore, this study aimed at developing a PCR-based assay for the detection of *M. hyopneumoniae* mRNA and at validating its applicability to differentiate viable from non-viable bacteria. Development of the RNA-based PCR encompassed studies to determine its analytical sensitivity, specificity, and repeatability, as well as its diagnostic accuracy. Comparisons between DNA and mRNA detection for the same target gene were performed to evaluate the ability of the RNA-based PCR to detect exclusively viable *M. hyopneumoniae* after bacterial inactivation using various methods. The RNA-based PCR was also compared to the DNA-based PCR as a tool to monitor the growth of *M. hyopneumoniae in vitro*. Under the conditions of this study, the developed RNA-based PCR assay detected only viable or very recently inactivated *M. hyopneumoniae*, while the DNA-based PCR consistently detected cells irrespective of their viability status. Changes in growth activity over time were only observable via RNA-based PCR. This viability PCR assay could be directly applied to evaluate the clearance of *M. hyopneumoniae* or to determine the viability of the bacterium at late stages of eradication programs.

4.2 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary etiologic agent of enzootic pneumonia (Goodwin et al., 1965; Mare & Switzer, 1965), a prevalent disease in the porcine industry worldwide, which causes important effects on animal health, welfare, and productivity (Pieters & Maes, 2019). The disease is clinically characterized by non-productive cough of variable severity and duration, high morbidity, and low mortality. *Mycoplasma hyopneumoniae* is also a primary pathogen of the Porcine Respiratory Disease Complex, a multifactorial respiratory condition for which this bacterium is considered to facilitate the participation of other viral and bacterial agents (Saade et al., 2020). To mitigate the detrimental effect of *M. hyopneumoniae* infection, the adoption of control measures, such as disease elimination or pathogen eradication, are being increasingly implemented, especially in North America. Disease control frequently involves changes in management practices, vaccination, and/or antibiotic treatments (Maes et al., 2018). Nevertheless, to monitor the efficacy of disease control measures and to investigate the causes of eradication failures, an accurate diagnosis is crucial.

Research has improved the capacity to accurately diagnose the disease caused by *M. hyopneumoniae* by obtaining more sensitive sample types or by developing assays with increased sensitivity, such as PCR (Garcia-Morante et al., 2022). However, further aspects of *M. hyopneumoniae* diagnostics would benefit from improvement in order to aid veterinary practitioners in their decision-making process in the field. One of the main diagnostic aspects to refine is the assessment of the viability status of *M. hyopneumoniae* that is detected by PCR in clinical specimens. PCR assays for *M. hyopneumoniae* detect DNA derived from live and dead bacterial cells and thus, cannot be used to identify viable (potentially infectious) cells or to assess the metabolic activity of the detected genetic material (Lauri & Mariani, 2009).

The most used methods to assess bacterial viability are culture-dependent. *Mycoplasma hyopneumoniae* is known for being extremely fastidious to grow in laboratory conditions (Kobisch & Friis, 1996), thus hindering the applicability of culture as a viability indicator. Culture-independent methods, such as viability PCR assays using dyes like ethidium monoazide (EMA), can be used as a tool for selective detection of nucleic acids from live bacterial cells (Rudi et al., 2005). Nevertheless, results obtained in a pilot study from our research group indicated the lack of suitability of the method for *M. hyopneumoniae*, as EMA was taken up and intercalated in all samples, regardless of known viability status and photoactivation settings (Anderson et al., 2015). Conversely, bacterial messenger RNA (mRNA) has been proposed as an indicator for cell viability, as half-lives of mRNAs can vary from seconds to minutes, their production ceases after cell death and are prone to degradation in the environment (S. S. Kumar & Ghosh, 2019). The development of PCR assays targeting mRNAs of constitutively conserved genes is a culture-independent method that has been used for the exclusive detection of viable bacterial cells (Lleò et al., 2000). However, a viability PCR assay is not available for the detection of *M. hyopneumoniae*.

The inability to assess bacterial viability is a key limiting factor for the diagnosis of *M. hyopneumoniae*, especially when assessing the efficacy of disease control interventions in the field, such as pathogen eradication, that rely on the duration of pathogen persistence post-infection. Recently, longer than expected persistence of pathogen detection by PCR post-exposure has been described, although the viability of the detected genetic material could not be ascertained (McDowell et al., 2023). Therefore, the objective of this study was to develop a PCR-based assay for the detection of *M. hyopneumoniae* mRNA and validate its applicability to differentiate viable from inert bacteria.

4.3 Materials and Methods

A diagram describing the main steps followed in the study is presented in Figure 4.1. For the purpose of this manuscript, real-time PCR will be referred to as DNA-based PCR and real-time, reverse-transcriptase PCR will be referred to as RNA-based PCR.

4.3.1 Development and validation of a PCR for mRNA detection of M. hyopneumoniae

4.3.1.1 Bacterial genetic material purification

Mycoplasma hyopneumoniae strain 232 (purchased from Iowa State University, Ames, IA) was the primary bacterial isolate used for this study. DNA from the bacterium was extracted by using MagMAX™ CORE Nucleic Acid Purification Kit coupled with MagMAX^{TM} Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). For total RNA extraction, Qiagen RNeasy universal mini kit (Qiagen, Dusseldorf, Germany) was used. Genomic DNA removal from the extracted total RNA was performed using TURBO DNA-*free*™ Kit (Thermo Fisher Scientific). Absence of genomic DNA contamination was confirmed via PCR (no RT control). Extracted DNA and RNA were stored at -80ºC immediately post extraction and were tested after only one freeze-thawing cycle to prevent degradation of nucleic acids.

4.3.1.2 PCR assay design

The *M. hyopneumoniae* gene *mhp*165 (genome NC_006360.1; Minion et al., 2004), a highly conserved, large (6,140 bp) gene, was the selected target for the present assay, as it showed to be constitutively present in a vast array of genetically diverse *M. hyopneumoniae* strains in a previously developed real-time PCR assay (Strait et al., 2008). Forward and reverse primers and a ZEN double-quenched probe containing a 5' FAM fluorophore, a 3' Iowa Black FQ quencher, and an internal ZEN quencher were designed using Prime Quest software (Integrated DNA Technologies, Coralville, IA; Table 4.1). The sequences were *in silico* evaluated using the PrimerBlast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to rule out homology to other agents (Bustin & Huggett, 2017). The forward and reverse primers, at a final concentration of 400 nM, and the probe, at a final concentration of 120 nM, were added to the DNA-based or RNA-based master mix (VetMAX™-Plus qPCR Master Mix or VetMAX™-Plus One-Step RT-PCR Kit, respectively; Thermo Fisher Scientific, Waltham, MA) in a 17 µL volume and were combined with 8 µL of template. The ABI 7500 Fast real-time PCR platform (Thermo Fisher Scientific) was used with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec for the DNA-based PCR and 48°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec for the RNA-based PCR, using the standard cycling mode.

Routine controls were included in each assay run, including an exogenous internal control RNA (XIC; VetMax™ Xeno RNA™, Thermo Fisher Scientific), a quantified, targeted synthetic oligonucleotide as positive amplification control (PAC; Gblocks ™ Gene Fragments, Integrated DNA Technologies) and no template controls.

4.3.1.3 *Assay validation*

Assay validation followed the guidelines of the Laboratory Technology Committee of the American Association of Veterinary Diagnostic Laboratories (Toohey-Kurth et al., 2020) and encompassed analytical and diagnostic performance, and repeatability, using reference strains and clinical specimens commonly collected for pathogen detection.

4.3.1.3.1 *Analytic accuracy*

Analytical sensitivity: Serial 10-fold dilutions spanning eight orders of magnitude were prepared from the PAC. The assay limit of detection (LOD) was calculated based on the mean of the Ct value at the lowest copy number wherein 100% of the replicates were detected positive. The LOD was established based on the results of 11 replicates in six independent runs.

Analytical specificity: One *M. hyopneumoniae* reference strain (ATCC 25095) and three field isolates (obtained from pigs with enzootic pneumonia) were tested to evaluate the ability of the assay to detect relevant strains (inclusivity). A collection of 32 pathogens, including 12 Mycoplasma species of pigs, birds, rodents, and humans, as well as 12 other bacterial species and nine swine viruses were inspected to prove the ability of the assay to distinguish from genetic near-neighbors and porcine respiratory microflora (exclusivity;

Table 4.2). Four different clinical specimen matrices, including nasal, laryngeal, tracheal and bronchial secretions, both from experimentally infected and negative pigs were collected in RNAlater® stabilization solution (Thermo Fisher Scientific) and evaluated to determine a hypothetical susceptibility to specimen matrix inhibitors (selectivity). In order to evaluate the preservation of mRNA in the clinical specimens, a PCR assay containing primers and probes targeting host mRNA, specifically porcine beta-actin mRNA, was used as an endogenous internal control (Prime PCR™, Bio-Rad Laboratories, Hercules, CA).

Repeatability: To measure the degree of agreement among replicates of a sample within and between runs, three concentrations of extracted RNA (high, medium, and low) were performed via serial dilutions, and five replicates of the concentrations (intra-assay variation) were tested in six separate runs (inter-assay variation).

4.3.1.3.2 *Diagnostic accuracy*

To determine diagnostic sensitivity (i.e., the proportion of known infected reference pigs that test positive) and diagnostic specificity (i.e., the proportion of non-infected reference pigs that test negative), a set of well-characterized clinical samples was obtained. It was composed of samples from pigs with known infectious status, obtained from two previous experimental infection studies with *M. hyopneumoniae.* All clinical samples had been previously tested using a commercially available PCR assay (Strait et al., 2008). In the first experimental infection, pigs were intratracheally inoculated with a lung homogenate containing a field strain of *M. hyopneumoniae* (McMahon et al., 2020). In the second experiment, pigs were infected via aerosol of a lung homogenate containing *M. hyopneumoniae* 232 (Garcia-Morante, De Abreu, et al., 2022). Clinical specimens from 23 infected and four non-infected control pigs were preserved, either in PBS or in RNAlater®, and tested by DNA-based PCR or RNA-based PCR, respectively.

4.3.2 Comparison of the DNA-based PCR and RNA-based PCR assays on the detection of viable and inactivated M. hyopneumoniae cells in vitro

The detection dynamics of DNA and mRNA in both viable and inactivated *M. hyopneumoniae* cells over time was performed with a side-by-side comparison using viable and inactivated cultures. Inactivated cultures were treated by either chemical or heat-based methods. Briefly, 150 mL of a *M. hyopneumoniae* stock culture, grown at 37°C in agitation at 100 rpm, was aliquoted and allotted into four treatments. In treatment 1, the culture was maintained under standard incubation conditions at 37°C and agitation at 100 rpm. In treatment 2, the culture was maintained at room temperature (25°C). In treatment 3, the culture was inactivated by adding formaldehyde at a 1:10 ratio (1 volume of formalin for 9 volumes of culture) and maintained at room temperature. In treatment 4, the culture was inactivated via autoclaving for 40 min at 121°C and 15 psi and then maintained at room temperature. Samples were obtained from the stock culture prior to aliquoting and 1, 2, 6 and 12 hours, and 1, 2, 3, 5, 10 and 20 days after treatments were started. At each timepoint, one 300 μL subsample was collected for DNA extraction and two, 1 mL samples were collected and placed in 0.5 mL of RNAlater® for RNA extraction. All DNA and RNA samples were analyzed by DNA-based PCR and RNA-based PCR, respectively.

To accurately define the detection decay dynamics of mRNA in the first minutes after inactivation, a separate longitudinal experiment was performed including the two cultures inactivated via the aforementioned conditions and monitoring the detection of DNA and RNA immediately prior to and 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 minutes post-inactivation. In this case, RNAlater® was not added to the samples collected for RNA extraction, and all DNA and RNA samples were also analyzed by DNA-based PCR and RNA-based PCR, respectively.

4.3.3 Assessment of M. hyopneumoniae growth in vitro by monitoring the transcriptional activity by RNA-based PCR

The developed RNA-based PCR assay was applied at different timepoints on a freshly grown *M. hyopneumoniae* culture in order to monitor the growth/metabolic activity of that specific bacterial community *in vitro*. A log-phase *M. hyopneumoniae* 232 stock culture was passaged at a 1:100 dilution in Friis-based culture medium (Mycoplasma Experience, Surrey, UK) to a final volume of 14 mL and incubated at 37°C in an orbital shaker at 100 rpm. Samples were collected from the stock culture immediately prior to passage and daily from day 0 to 7 days post-passage. Collected samples were subject to DNA and RNA extraction for DNA-based PCR and RNA-based PCR testing respectively, the latter performed in two replicates. Additionally, pH was monitored daily and compared to culture medium kept under the same conditions of the culture.

4.4 Results

4.4.1 Assay validation

The analytical limit of detection was one bacterial genome copy equivalent/µL, which corresponds to eight genome copy equivalents per assay reaction (Table 4.3). All tested *M. hyopneumoniae* strains were detected with the newly developed assay (inclusivity), and no other Mycoplasma species were detected. No detection was obtained from the extensive porcine bacterial and viral panel of targets (data not shown). Additionally, the target was detected from all the evaluated clinical specimen matrices from *M. hyopneumoniae* experimentally infected pigs. The target was not detected in samples from non-infected pigs (data not shown).

Regarding the repeatability of the assay, there was very low intra- and inter-assay variation, as all the SD were <1 (Table 4.4).

4.4.2 Comparison between DNA-based PCR and RNA-based PCR assays on the detection of viable and inactivated M. hyopneumoniae cells in vitro

Mycoplasma hyopneumoniae DNA was consistently detected by DNA-based PCR irrespective of the viability status, and the relative quantity of the target did not differ over time after inactivation (Figure 4.2A). In the cultures inactivated via autoclaving or formaldehyde addition, there was a reduction of the relative quantity of target, with a Ct value increase of approximately 2 logarithmic values immediately post-inactivation, which was visible on the first timepoint after treatment. Ct values remained stable after inactivation.

Mycoplasma hyopneumoniae mRNA was not detected by RNA-based PCR in non-viable cells inactivated via either of the two methods (i.e., formaldehyde addition and autoclaving) (Figure 4.2B) on the first hour post-inactivation and onwards. In the two treatments in which cells were not inactivated, RNA detection remained largely unchanged over time for 20 days post-inactivation (last timepoint measured), although with variable Ct values, depicting an apparent wave-like pattern.

Mycoplasma hyopneumoniae mRNA was not detected at any timepoint within the first hour post-inactivation via autoclaving. In the formaldehyde-inactivated culture, there was a gradual increase in Ct value over time until 25 min post-inactivation, at which point mRNA was no longer detected (Figure 4.2C). The XIC was detected at constant levels throughout time, revealing no inhibition effects in the samples. Similar to what was observed previously, DNA was detected at a steady level by DNA-based PCR.

4.4.3 Assessment of M. hyopneumoniae growth in vitro by monitoring the transcriptional activity by RNA-based PCR.

Prior to passage, the DNA and mRNA detection levels of the log-phase *M. hyopneumoniae* culture did not significantly differ in the stock culture. Immediately post-passage, there was a Ct value increase in the DNA-based PCR, likely caused by a dilution effect. The Ct value in the RNA-based PCR was steady on day zero post-passage and evidenced a marked decrease in the first two days post-passage, being approximately four Ct values lower than the Ct value of the DNA-based PCR at day 2 post-passage. From day two to seven post-passage, the Ct values of the DNA-based PCR remained constant within one Ct value difference between days. Conversely, Ct values of the RNA-based PCR showed a gradual tendency to increase, with a difference of ten Ct values between day two and day seven post-passage (Figure 4.3A).

In parallel, daily pH measurements of the growing culture indicated a gradual acidification beginning on day one through day five post-passage, remaining steady thereafter until day seven. In the uninoculated culture medium maintained in the same conditions of the passaged culture, a tendency to basification was observed (Figure 4.3B).

4.5 Discussion and Conclusions

A PCR assay based on the detection of *M. hyopneumoniae* mRNA was developed in this study. The assay was applied to culture samples of known viability status to demonstrate its ability to differentiate between viable and non-viable *M. hyopneumoniae* cells. Under the conditions of this study, the developed RNA-based PCR assay detected only viable or very recently inactivated *M. hyopneumoniae*. The mRNA detection of inactivated *M. hyopneumoniae* gradually declined within the first 25 minutes post-inactivation, becoming undetectable after that time.

The half-life of mRNA in the *Escherichia coli* bacterial model typically varies between three and eight minutes (Chen et al., 2015; Esquerré et al., 2014). The high turnover rates and rapid decay after production are fundamental characteristics for the development of gene expression studies aiming at understanding cell cycle metabolism, signaling pathways and, in definitive, the physiology of prokaryotic and eukaryotic cells. These features, however, have also been considered for the use of mRNA as a viability indicator and therefore, RNA-based PCR assays have been developed for the exclusive detection of viable bacterial species (Emerson et al., 2017; McIngvale et al., 2002; Zhou et al., 2014). Although the application of RNA-based PCR to enumerate viable *M.*

hyopneumoniae cells has not been previously explored, an RNA-based PCR assay to assess the viability of *Mycoplasma synoviae*, a poultry pathogen that causes respiratory disease and decreased egg production, has been reported (Marois et al., 2005). In that study, *M. synoviae* mRNA was detected by RNA-based PCR up to 20 minutes post-inactivation via osmotic shock. This result coincides with what was observed in the present study, in which the mRNA of *M. hyopneumoniae* was detected up to 25 minutes post-chemical inactivation. Furthermore, in another study using mRNA detection by RNA-based PCR to determine the viability status of *Salmonella enterica*, the authors used the assay on heat-treated cells at 121°C and were not able to detect mRNA, evidencing that the RNA-based PCR system had the capability to detect only viable *Salmonella* (Zhou et al., 2014). However, in another study using different lethal treatments such as desiccation stress or isopropanol exposure, *E. coli* O157:H7 cell numbers declined 10^7-fold within 96 hours according to culture-based assessments, although there was only a 100-fold reduction in *E. coli* levels according to an RNA-based PCR assay (Ju et al., 2016).

A remarkable finding derived from the present study was the extended detection of mRNA in the non-inactivated *M. hyopneumoniae* cultures, that is, cultures that were maintained at standard incubation conditions and at room temperature, in which mRNA was detected until 20 days post-treatment, the last time of observation. The extended survival of *M. hyopneumoniae* in culture, without the addition of fresh culture medium, observed in this work, agrees with the results of a previous study on the survivability (or viability) of *M. hyopneumoniae* determined by subsequent growth in liquid medium

(Goodwin, 1972). In that study, *M. hyopneumoniae* survived 18 to 31 days in liquid medium at room temperature, and seven to 14 weeks in liquid medium at refrigeration temperatures. The survival time in solid medium at 37°C was at least 20 days and, in small pieces of pneumonic tissue, the survival time was seven days at room temperature and at least 11 days at 4°C. In addition, the survivability of different porcine Mycoplasmas to drying has also been investigated (Friis, 1973). *Mycoplasma hyopneumoniae* resisted between six and eight days under extremely low humidity conditions. The *in vitro* survival of *M. hyopneumoniae* on different dry surfaces encountered in pig farms at various temperatures was up to several days, again determined by culture-based assessment, in a more recent study (Browne et al., 2017). In all these previous experiments, the viability of *M. hyopneumoniae* was established via bacterial culture, a methodology known to have low sensitivity for this bacterium. Thus, the developed viability assay can be used to generate additional, and potentially more accurate data on the survivability of *M. hyopneumoniae* in different environmental conditions. This tool would be essential to investigate the capacity of indirect transmission to pigs from the environment or fomites, as well as the efficacy that disinfecting products routinely used in farms or transport vehicles, have on the viability of *M. hyopneumoniae*.

The viability assay developed in this investigation can also provide crucial information to better understand complex host-pathogen interactions in different scenarios. These include the determination of the efficacy in bacterial clearance post-antibiotic treatment with different types of antibiotics, or the presence of viable *M. hyopneumoniae* and their metabolic status in chronic stages of disease, when the immune system of the host and the virulence factors of the bacterium compete in the infection process.

In addition, in this study, *M. hyopneumoniae* mRNA detection was compared to DNA detection and pH to monitor the growth dynamics of a culture in the first seven days post-passage, similar to the use of optical density measurements to construct growth curves in other bacterial species. While DNA detection by DNA-based PCR increased the two first days post-passage, the degree of difference was not remarkable and it remained constant after. In contrast, mRNA detection by RNA-based PCR increased significantly in the first two days, with slightly more than one logarithmic difference in detection between DNA and mRNA. After that, the increase in mRNA detection was still very substantial up until day seven post-passage. The experiment showed the ability of the newly developed RNA-based PCR assay to capture the differences in growth dynamics and kinetics of *M. hyopneumoniae in vitro* in standard conditions, while differences in metabolic activity were not detected by PCR.

RNA-based PCR Ct values can be lower than DNA-based PCR Ct values for the same gene due to the fact that transcription of multiple mRNA transcripts of a single gene occur when there is increased need for the protein(s) encoded by that gene, such as when the cell is at increased metabolic activity. Conversely, there is down-regulation of mRNA transcription when the need for the protein(s) encoded by a particular gene is reduced. Thus, mRNA detection by RNA-based PCR can be added to other laboratory techniques used to monitor the *in vitro* growing dynamics of different *M. hyopneumoniae* strains, such as the color changing units assay (Stemke & Robertson, 1982), the colony forming units assay (Garcia-Morante et al., 2018; Stemke & Robertson, 1982), ATP luminometry (Calus et al., 2010; Garcia-Morante et al., 2018), or flow cytometry (Assunção et al., 2005). By combining different techniques, strains with distinctive growth performance could be identified *in vitro*, which would eventually infer distinctive virulence or pathogenic potential *in vivo* and be selected as potential vaccine candidates or for controlled exposure purposes.

Moreover, the assessment of the metabolic or growing activity of *M. hyopneumoniae* using RNA-based PCR can shed light on the possibility of describing the presence of persister cells for this bacterium, defined as non- or slow- dividing cells that are not antibiotic-resistant but tolerate higher levels of antibiotics compared to other cells (Balaban et al., 2019). Also, it can help determining if *M. hyopneumoniae* can adapt to conditions of stress or starvation, either *in vitro* or *in vivo*, through entering a state of dormancy (Lempp et al., 2020) or adopting a very low metabolic state known as viable but non-culturable state (Coutard et al., 2005; Dong et al., 2020). It is presumable that the characteristics of the environment in which bacteria survive have a great impact on their viability and growth status. Thus, one of the limitations of the present study is that only two methods of bacterial inactivation were used. Therefore, additional mechanisms of inactivation based on the development of environmental conditions or substances lethal to bacteria should be investigated.

An accurate test is a critical component of diagnostic investigations aiming at detecting a pathogen and controlling different aspects of the epidemiology of the diseases that it causes. However, diagnostic methods can be very sensitive, hence challenging to interpret in the context of the entire clinical-pathological scenario. An example of the latter can be PCR assays for the detection of a particular pathogen in a sample. For routine diagnostic purposes, the presence of *M. hyopneumoniae* in a clinical sample is determined by PCR targeting bacterial DNA, as DNA molecules are very stable and resistant to degradation even when subject to harsh environmental conditions. Although this characteristic is beneficial, for example, for retrospective diagnostic investigations using archived samples or when the time and between sample collection and testing is long and the circumstances are not ideal, it can also be confusing, since DNA of inactive cells may be targeted and amplified by PCR. Thus, it is important to remember that there is no direct relationship between viability or metabolic status of bacteria and their DNA detection by PCR, which in conjunction to the inherent difficulty to isolate *M. hyopneumoniae* in bacterial culture, can lead to result interpretation issues. Therefore, methods to determine the viability status of *M. hyopneumoniae* other than culture have been a historical need. Thus, the applicability of the PCR-based viability assay described in this study will be fundamental for understanding several aspects of the pathogenesis, epidemiology, and control of this pathogen.

Table 4.1 Primer and probe sequences used for the DNA-based and RNA-based PCR assays.

Primer/probe	Sequence	Amplicon size (bp)
mhp165 Forward	5'- AAAGCCTCCTCGCCATTT-3'	100 bp
mhp165 Reverse	5'- CCTGGGCATAATTTAGTGTTTGG-3'	
mhp165 Probe	5’-6-FAM-TGTTGGCGC/ZEN/TCATGAATATGGTCATCA-IABkFQ-3’[a]	

[a] FAM, 6-carboxyfluoroscein; ZEN, internal quencher; IABkFQ, Iowa Black Quencher.

Table 4.2 Pathogens used for analytical specificity determination.

Bacteria	Viruses
Mycoplasma hyopneumoniae (232; reference strain)	PCV type 1*
Mycoplasma hyopneumoniae (AP1215-2; field strain)[†]	PCV type 2*
Mycoplasma hyopneumoniae (AP216-1; field strain)[†]	PRRSV type 1*
Mycoplasma hyopneumoniae (AP417; field strain)[†]	PRRSV type 2*
Mycoplasma hyopneumoniae (ATCC 25095; Mare and Switzer)	Influenza A virus*
Mycoplasma hyorhinis (ATCC 17981)	Porcine cytomegalovirus*
*Mycoplasma hyosynoviae**	PRCV *
Mycoplasma flocculare (ATCC 27716)	
*Mycoplasma bovis**	
Mycoplasma gallisepticum (ATCC 19610)	
*Mycoplasma gallinarum**	
*Mycoplasma synoviae**	
Mycoplasma cloacale (ATCC 35276)	
*Mycoplasma pullorum**	
Mycoplasma arginini (ATCC 23838)	
Mycoplasma pneumoniae (ATCC 15531)	
Mycoplasma orale (ATCC 23714)	
Mycoplasma pulmonis (ATCC 19612)	
*Streptococcus suis**	
*Bordetella bronchyseptica**	
*Glaesserella parasuis**	
*Pasteurella multocida**	
*Actinobacillus pleuropneumoniae**	
*Actinobacillus suis**	
*Escherichia coli**	
*Trueperella pyogenes**	
*Bacillus cereus**	
*Actinobacillus porcinus**	
*Salmonella spp**	
*Staphylococcus aureus**	

PCV: Porcine Circovirus

PRRSV: Porcine Reproductive and Respiratory Virus

PRCV: Porcine Respiratory Coronavirus

* Kindly provided by the Veterinary Diagnostic Laboratory of the University of Minnesota.

[†] Sourced from internal isolate collection.

Table 4.3 Limit of detection based on 10-fold dilutions of a quantified, targeted synthetic *mhp165* oligonucleotide.

Replicate/Dilution	100000*	10000	1000	100	10	1	0.1	0.01
1	19.16	22.64	26.01	29.47	32.37	33.61	Negative	36.18
2	20.91	23.49	26.06	29.09	31.91	33.40	38.15	37.85
3	20.42	23.18	26.19	29.11	32.24	32.82	37.96	37.32
4	19.19	22.65	26.04	29.38	32.57	33.51	Negative	37.74
5	19.15	22.60	26.11	29.27	32.29	33.44	Negative	38.58
6	19.34	22.82	26.22	29.61	33.03	36.33	39.67	Negative
7	19.40	22.83	26.27	29.75	33.20	37.33	38.06	Negative
8	19.30	22.83	26.08	29.72	32.58	35.14	36.76	Negative
9	21.26	25.59	26.40	29.69	32.59	36.98	Negative	Negative
10	19.24	22.96	26.74	30.57	33.73	36.90	Negative	39.10
11	19.28	22.96	26.69	30.91	33.53	36.75	37.69	38.00
Ct value (Mean ± SD)	19.70 ± 0.78	23.14 ± 0.85	26.26 ± 0.25	29.69 ± 0.57	32.73 ± 0.57	35.11 ± 1.77	38.05 ± 0.94	37.82 ± 0.93

* Genome copy number/µL.

Results expressed as Ct values.

Table 4.4 Repeatability determination of the RNA-based PCR assay.

	Target concentration		
Replicate	Low	Medium	High
A	24.24*	28.58	31.96
	24.34	28.59	32.06
	24.24	28.62	32.06
	24.30	28.59	32.27
	24.25	28.57	32.21
B	24.20	28.68	32.25
	24.26	28.71	32.05
	24.31	28.67	32.17
	24.24	28.72	32.05
	24.38	28.70	32.21
C	24.33	28.74	32.08
	24.34	28.87	32.17
	24.34	28.81	32.27
	24.35	28.78	32.08
	24.36	28.87	31.85
D	24.37	28.76	32.21
	24.30	28.86	32.28
	24.33	28.80	32.00
	24.49	28.80	32.13
	24.38	28.83	31.99
E	24.33	28.82	32.40
	24.37	28.85	32.27
	24.41	28.84	32.09
	24.40	28.79	32.12
	24.35	28.83	31.86
F	24.30	28.79	32.49
	24.24	28.96	32.67
	24.36	28.85	32.40
	24.55	28.93	33.29
	24.25	28.91	32.11
Ct value (Mean ± SD)	24.33 ± 0.08	28.77 ± 0.11	32.20 ± 0.27

Five replicates of three different concentrations of the target gene were run in six different testing events.

*Results expressed as Ct values.

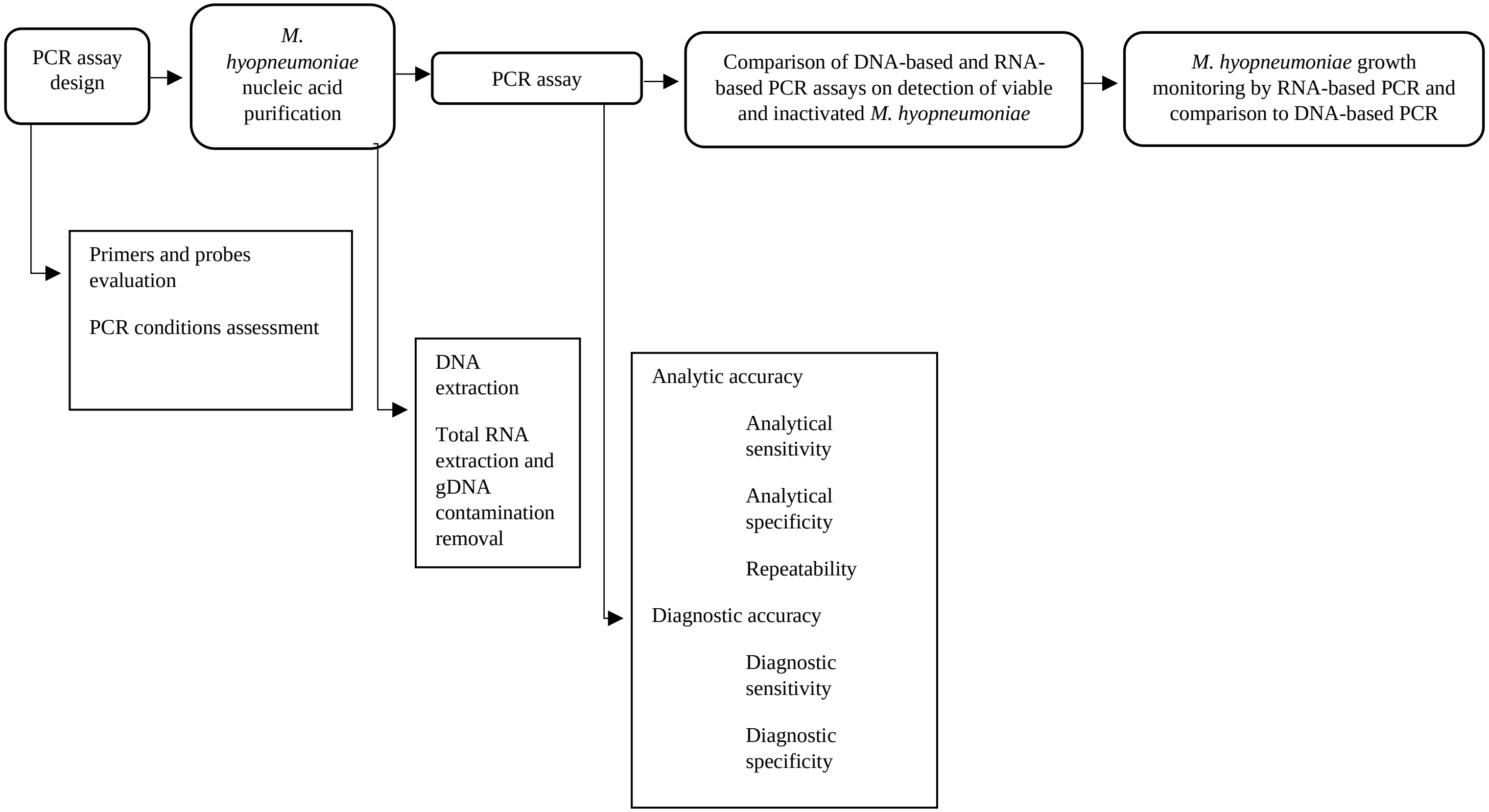

Figure 4.1 PCR assay development workflow.

The development and validation of the PCR involved assay design and validation and nucleic acid extraction. When validated, the assay was used assess the discriminate detection of viable and inactivated *Mycoplasma hyopneumoniae* and to monitor the growth of cultured *Mycoplasma hyopneumoniae*.

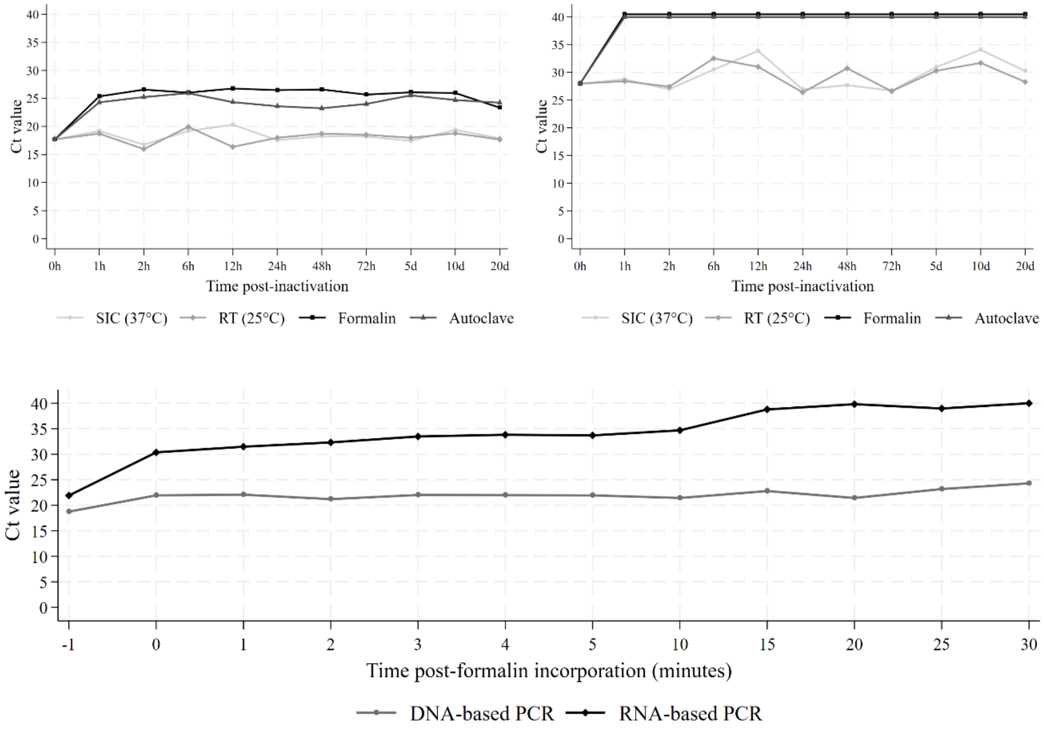

Figure 4.2 Comparison between DNA and mRNA detection of *M. hyopneumoniae* cultures with different viability statuses.

A) DNA detection by DNA-based PCR. B) mRNA detection by RNA-based PCR. C) DNA and mRNA detection decay within 30 minutes post-inactivation via formalin incorporation by DNA-based PCR and RNA-based PCR, respectively. SIC = Standard incubation conditions. RT = Room temperature

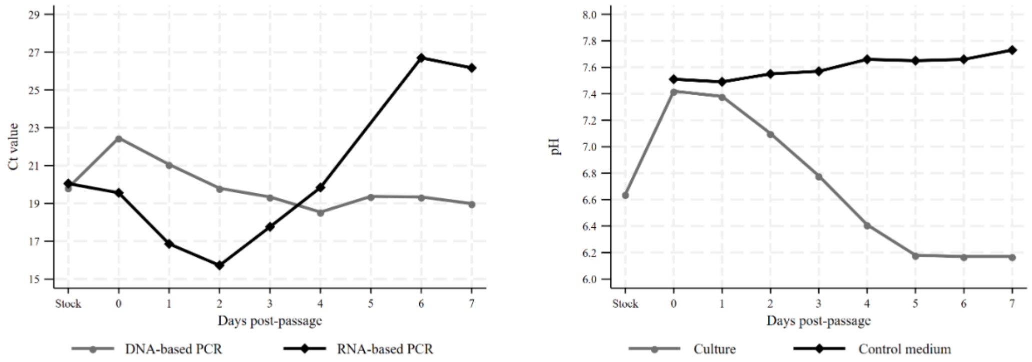

Figure 4.3 Growth activity of *M. hyopneumoniae* post-passage.

Stock indicates the log-phase culture from which a 1:100 dilution passage was performed, at day 0 post-passage. A) DNA and mRNA detection by DNA-based PCR and RNA-based PCR, respectively. Note that RNA-based PCR was not performed on D5 post-passage. B) pH measurements. A non-inoculated culture medium maintained at the same conditions as the passaged culture was used for comparison purposes.

Chapter 5: Evaluation of *Mycoplasma hyopneumoniae* viability in antibiotic-treated pigs under commercial conditions

5.1 Summary

Several measures, such as antibiotic treatment, can be put in place to control and reduce the detrimental effects that the infection with *M. hyopneumoniae* causes on pigs. The positive effects of treatment can be measured using different metrics, which can be based on clinical, pathologic, or laboratorial outcomes. Among the latter, genetic material detection by PCR is the most employed method for this purpose. However, the interpretation of PCR detection of *M. hyopneumoniae* can be especially challenging in the context of antibiotic treatment, as PCR assays for *M. hyopneumoniae* are based on total bacterial DNA detection, and thus, the viability of the detected genetic material cannot be ascertained. Therefore, the objective of this study was to evaluate the viability of *M. hyopneumoniae* post-antibiotic treatment in experimentally infected and treated pigs under commercial conditions. A total pf 42 pigs were inoculated with *M. hyopneumoniae* and PRRSV and, 20 days post-inoculation (dpi), pigs were treated with tilmicosin and oxytetracycline for ten consecutive days. Four sampling events were defined: 1) pre-inoculation, pre-treatment (-42 dpi); 2) post-inoculation, pre-treatment (12 dpi/-8 days post-treatment (dpt)); 3) post-inoculation, first post-treatment (33dpi/13dpt) and 4) post-inoculation, second post-treatment (46 dpi/26dpt). Collected tracheal secretions were subject to both DNA- and RNA-based PCR testing. There were statistically significant quantitative differences between the detection of *M. hyopneumoniae* DNA and mRNA at all timepoints when the bacterium was detected, regardless of antibiotic treatment, suggesting that DNA-based PCR may overrepresent the viable *M. hyopneumoniae* population. Additionally, the difference in mean Ct value

between the two PCR assays was higher in the second post-treatment sampling, compared to the first, suggesting that the difference between the detection of viable-only and total *M. hyopneumoniae* may be increased post-antibiotic treatment. Results from this study suggest that the use of diagnostic methods that allow the relative quantification of viable cells may be more suitable to determine the efficacy of antibiotic treatments than DNA-based methods. However, the clinical and disease control implications of the observed differences are currently unknown and should be investigated in further research efforts.

5.2 Introduction

Mycoplasma hyopneumoniae is the primary pathogen involved in the chronic respiratory condition of pigs known as enzootic pneumonia, and is one of the key agents associated with the Porcine Respiratory Disease Complex (PRDC). The disease caused by *M. hyopneumoniae* harbors high and worldwide prevalence, and is characterized by coughing and reduced growth performance in finishing pigs (Pieters & Maes, 2019), frequently requiring antibiotic treatment.

Several measures can be put in place to control and reduce the detrimental effects that the infection with *M. hyopneumoniae* causes on pigs. Production management practices, such as acclimation of replacement gilts or internal biosecurity procedures can reduce the infection pressure and improve the clinical picture in endemic farms (Marco et al., 2020). Another frequent control strategy for *M. hyopneumoniae* is vaccination, which can reduce lung lesion development and bacterial shedding in infected animals, although it does not prevent colonization and/or infection (Maes et al., 2021). Antibiotic treatment is required when the clinical disease exists to prevent economic losses due to decreased performance and to protect animal welfare and wellbeing. Additionally, antimicrobials are frequently employed in *M. hyopneumoniae* elimination programs (Yeske et al., 2020). Similar to vaccination, antibiotic medication can help at reducing the clinical signs, lesions and performance impact associated to *M. hyopneumoniae* infections (Goodwin, 1979) without inducing total cure. Several different types of antimicrobials are effective against *M. hyopneumoniae*, although tetracyclines and macrolides are perhaps the most frequently used, as they are also effective against other secondary bacterial pathogens involved in PRDC (Maes et al., 2020).

As with several disease control interventions, it is essential to assess the effectiveness or impact of a particular strategy on the clearance of a pathogen from the host. The positive effects of treatment can be measured using different metrics, which can be of clinical or pathologic nature (Garcia-Morante et al., 2016; Nathues et al., 2010). In addition, laboratory-based methods, such as genetic material detection by PCR and bacterial isolation, can also be employed for this purpose.

Nevertheless, the interpretation of PCR detection of *M. hyopneumoniae* can be especially challenging in the context of antibiotic treatment, as persistence of bacterial detection post-treatment has been described for different types of antibiotics, such as tulathromycin (Betlach et al., 2021; Painter et al., 2012), chlortetracycline (Thacker et al., 2006) or marbofloxacin (Le Carrou et al., 2006), by PCR. However, PCR assays for *M. hyopneumoniae* are based on total bacterial DNA detection, and thus, the viability of the detected genetic material cannot be ascertained (Josephson et al., 1993). For instance, in a study by Betlach et al., (2021), *M. hyopneumoniae* genetic material was detected by PCR in gilts up to approximately three months after receiving antibiotic treatment. Despite that, transmission of the bacterium from these persistently infected to close-contact, naïve pigs was not observed, suggesting that PCR results could potentially represent the detection of non-viable cells.

Previous work by our research group, presented in chapter 3, has revealed lack of detection of inactivated *M. hyopneumoniae* post-inoculation, possibly indicating that in healthy pigs, the DNA of non-viable *M. hyopneumoniae* was rapidly sensed and cleared, being undetectable by PCR. However, pigs housed in field conditions can be subject to coinfections with pathogens, exposure to gases and aerosol particles that may impair the mucociliary apparatus and the phagocytic activity of immune cells and thus, could affect the capability of clearing cell detritus or pathogens.

Moreover, to evaluate the efficacy of antibiotic treatments against *M. hyopneumoniae*, it is essential to determine bacterial viability, although the remarkably low sensitivity of bacterial culture has been a limiting factor in this context (Chae et al., 2021; Friis, 1971). The recent development of a viability PCR assay for *M. hyopneumoniae*, described in chapter 4, is an alternative, culture-independent method to assess bacterial viability. Therefore, the objective of this study was to evaluate the viability of *M. hyopneumoniae* post-antibiotic treatment in experimentally infected and treated pigs under commercial conditions.

5.3 Materials and Methods

Animals and husbandry conditions

This study was performed in a 2,550-head, commercial, wean-to-finish site located in the Midwest U.S. The site layout consisted of 85 pens of 30 pigs each. Pigs were vaccinated at placement (three weeks of age) against Porcine Circovirus type 2, Porcine Reproductive and Respiratory Syndrome virus (PRRSV) and *M. hyopneumoniae* (3 FLEX, Boehringer-Ingelheim, Duluth, GA, USA) and against *Lawsonia intracellularis* (Enterisol Ileitis, Boehringer-Ingelheim, Duluth, GA, USA). A total of 42 pigs were randomly selected from 11 pens (two pigs per pen), individually identified and followed for sampling. All animal intervention protocols were approved by the Institutional Animal Care and Use Committee of the production company owning the pigs (protocol number 2021-22).

Experimental design

The experimental design of this study is depicted in Figure 5.1. At eight weeks of age, pigs were intratracheally inoculated (Gomes Neto et al., 2014) with ten mL of a lung homogenate containing $1\text{x}10^{5.5}$ color changing units/mL of *M. hyopneumoniae* strain 232 (purchased from Iowa State University, Ames, IA, USA) for two consecutive days. Seven days post-inoculation (dpi), pigs were intramuscularly inoculated with type 2 PRRSV (1 x $10^{4.9}$ TCID50 per 2mL dose, 2 mL per pig administered). Antibiotic and anti-inflammatory treatment started five days after the onset of the first coughing events (20dpi), recorded via an automated coughing monitoring device (SoundTalks®, Boehringer Ingelheim, Duluth, GA). Antibiotic treatment lasted ten days in total and consisted of the administration via water of tilmicosin (Pulmotil, Elanco, Fort Dodge, IA) for five consecutive days, followed by oxytetracycline (Pennox 343, Pharmgate, Wilmington, NC) via water for five consecutive days. Anti-inflammatory treatment consisted of the administration of a commercial sodium salicylate (Oral pro aspirin, Aurora pharmaceuticals, Northfield, MN) via water for ten consecutive days. All drugs were administered under veterinary supervision and at the labeled dosage for pigs.

Four sampling events were defined: 1) pre-inoculation, pre-treatment (-42 dpi); 2) post-inoculation, pre-treatment (12 dpi/-8 days post-treatment (dpt)); 3) post-inoculation, first post-treatment (33dpi/13dpt) and 4) post-inoculation, second post-treatment (46 dpi/26dpt).

Sample collection and processing

Tracheal secretions were collected from pigs as described by Fablet et al. (2010), with the exception that the sterile 5 mL tube in which the tip of the catheter was placed contained 1mL of RNAlater stabilization solution (Thermo Fisher Scientific, Waltham, MA). All collected samples were frozen until nucleic acid extraction was performed. Bacterial DNA was extracted by using MagMAX™ CORE Nucleic Acid Purification Kit coupled with MagMAX^{TM} Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). For total RNA extraction, Qiagen RNeasy universal mini kit (Qiagen, Dusseldorf, Germany) was used. Genomic DNA removal from the extracted total RNA was performed using TURBO DNA-*free*™ Kit (Thermo Fisher Scientific). Absence of genomic DNA contamination was confirmed via PCR (no RT control). Samples were tested via both real-time PCR (hereby referred to as DNA-based PCR) and real-time, reverse-transcriptase PCR targeting *M. hyopneumoniae* mRNA (hereby referred to as

RNA-based PCR), as described previously in chapter 4. All the samples that produced a Ct value (<40) were considered positive.

Data analysis

Differences in the mean Ct value between the DNA-based PCR and the RNA-based PCR for each sampling event were investigated via statistical paired t-test analysis. Additionally, PCR results, expressed as a binary dichotomous categorical variable (positive/negative), were tabulated in 2 x 2 contingency tables with DNA-based PCR in the columns and RNA-based PCR in the rows. Marginal homogeneity was determined using the non-parametric McNemar's chi square statistical test.

5.4 Results

Two pigs were lost to follow-up and were excluded from the analysis. The quantitative detection dynamics of *M. hyopneumoniae* DNA and mRNA using both types of PCR assays are shown in Figure 5.2. All pigs tested negative to *M. hyopneumoniae* prior to experimental inoculation. At the post-inoculation/pre-treatment sampling (12dpi/-8dpt), 38/40 (95%) pigs were positive by DNA-based PCR, with a mean Ct value of 29.38, while 37/40 (92.5%) pigs were positive by RNA-based PCR, with a mean Ct value of 31.95. At the first post-treatment sampling (43dpi/13dpt), 40/40 pigs were positive with both PCR types, with a mean Ct value of 26.37 and 28.39 for the DNA-based and RNA-based PCRs, respectively. At the second post-treatment sampling (46 dpi/26dpt), 40/40 (100%) pigs were positive by DNA-based PCR with a mean Ct value of 27.43 and 38/40 (95%) by RNA-based PCR with a mean Ct value of 29.97. There were statistically significant differences in mean Ct values between the DNA-based and the RNA-based PCRs for the post-inoculation/pre-treatment sampling (mean difference 2.63; 95% CI 1.99, 3.28; p-value = <0.001), the first post-treatment sampling (mean difference 2.01; 95% CI 1.33, 2.7; p-value = <0.001) and for the second post-treatment sampling (mean difference 3.04; 95% CI 2.01, 4.07; p-value = <0.001). There were no significant differences in marginal homogeneity between the two PCR assays at any sampling timepoint (Figure 5.3).

5.5 Discussion and Conclusions

In the present study, a comparison between *M. hyopneumoniae* detection by DNA-based PCR and RNA-based PCR was conducted before and after antibiotic treatment. The working hypothesis of this study was that by targeting viable-only cells, compared to targeting bacterial genetic material irrespective of its viability status, differences in bacterial clearance post-antibiotic treatment would be evidenced.

Data from this study showed statistically significant quantitative differences between the detection of *M. hyopneumoniae* DNA and mRNA at all timepoints when the bacterium was detected, regardless of antibiotic treatment. However, no qualitative differences, based on the categorical classification of results as positive or negative, were observed between the results obtained using the two assays. Results of this investigation suggest that assays designed to target total DNA of *M. hyopneumoniae*, such as the DNA-based PCR used in this study, may overrepresent the viable *M. hyopneumoniae* population. These results differ from the findings from a previous study, presented in chapter 3, on the detection of inactivated *M. hyopneumoniae* in the respiratory tract of healthy pigs. In that study, where pigs with non-compromised mucociliary apparatuses and immune systems were used, nucleic acids from dead cells were not detected at any timepoint in any of the respiratory and immune tissues evaluated. It can be hypothesized that cells of the immune system sensed the nucleic acids from dead cells and cleared them from the mucosal surface of the trachea. In the present study, pigs were subject to several factors commonly present in commercial pig operations, such as coinfections and exposure to gases, which are known to influence bacterial clearance (Toews, 1993). First, pigs had an active, acute infection with *M. hyopneumoniae*, a pathogen known to impair the mucociliary apparatus by attaching to and destroying the cilia of respiratory epithelial cells (Maes et al., 2018). Thus, if this mechanism is hindered, reduced clearance of residual nucleic acids from non-viable bacteria should be expected. Second, pigs were also infected with PRRSV, a pathogen with modulatory effects on immune cells, specifically decreasing the phagocytic activity of macrophages and potentially delaying or weakening the clearance of nucleic acids from the tracheal milieu (Chand et al., 2012). Finally, pigs in farm settings are exposed to variable levels of carbon dioxide, ammonia and dust, which can have a detrimental effect on the respiratory airways and the mucociliary apparatus (Toews, 1993). Under the conditions of this investigation, when the pigs were housed in settings that would mimic frequent health and environmental challenges, there appeared to be persistence of residual DNA detection of non-viable cells.

Results from this study also showed increases in bacterial load post-inoculation, as infection developed and progressively established. This was manifested in higher Ct values for both types of PCR assays in the pre-treatment compared to the post-treatment sampling events. A possible explanation for the higher bacterial load observed in the first sampling post-treatment would be that pigs were sampled when the bacterial load is expected to be highest (33 dpi and 46 dpi) compared to the initial stages of infection (12 dpi). These results are in accordance to the infection dynamics observed previously for *M. hyopneumoniae* (Roos et al., 2016). Also, the first post-treatment sampling occurred only three days after the treatment ended, when the effects of antibiotics on the viability of *M. hyopneumoniae* were probably still developing. Indeed, the potential effect of antibiotic treatment could be suggested by the observations of the second post-treatment sampling, as there was an increase in the mean Ct value for both PCR assays compared to the first post-treatment sampling, indicating a numerical reduction of the bacterial load in the respiratory tract of pigs. However, this can be only hypothesized, since the inclusion of an untreated control group prevents to determine if the observed changes are due to the effects of antibiotic or to the course of infection. The difference in mean Ct value between the DNA-based PCR and the RNA-based PCR was higher in the second post-treatment sampling compared to the first, suggesting that the difference between the detection of viable-only and total *M. hyopneumoniae* may be increased post-antibiotic treatment. Particularly for the RNA-based PCR, the observed increase of the mean Ct value post-treatment could be interpreted both as a reduction of the viable bacterial load or also as a decrease in the transcriptional activity of viable cells, that is, bacterial cells could still be viable, but metabolically less active.

The differences between the DNA and RNA-based PCR assays on the detection of *M. hyopneumoniae* post-treatment, although statistically significant, were biologically subtle, and this could be associated to several factors. First, both types of antibiotic compounds used in this study are classified as bacteriostatic and thus, they suppress the growth of bacteria by keeping cells in the stationary phase of growth rather than having a lethal effect (Pankey & Sabath, 2004). The mode of action of tetracyclines and macrolides is similar as both act by binding reversely to subunits of ribosomes (30S subunit for tetracyclines and 50S for macrolides), inhibiting protein synthesis (Baietto et al., 2014; Schwarz et al., 2016). In this scenario, even though the translation apparatus of bacteria is truncated, the transcription of mRNA through the DNA-dependent RNA polymerase is intact and thus, mRNA is continuously being produced by bacterial cells and eventually being detected by the RNA-based PCR assay used in this study. Potentially, the use of an antibiotic compound with the property of blocking only transcription could provide insights into the comparison of DNA and mRNA detection, both *in vitro* and *in vivo*. Rifampicin is an antibiotic that specifically blocks transcription by binding to the β subunit of RNA polymerase, preventing initiation of RNA synthesis (Campbell et al., 2001). This is one of the most potent and broad-spectrum antibiotics against bacterial pathogens and is a key component of anti-tuberculosis therapy. Unfortunately, Mycoplasma species are intrinsically resistant to rifampicin due to a natural mutation in the *rpoB* gene of the RNA polymerase β subunit, which prevents the antibiotic from binding its target (Gaurivaud et al., 1996). Therefore, although the use of this antibiotic compound could have been insightful for the *in vitro* model, its application in Mycoplasma species is limited. Also importantly, the use of rifampicin is restricted to humans and thus, no *in vivo* studies involving animals can be performed, even for susceptible bacterial species. Alternatively, the bactericidal antibiotic class fluoroquinolones (e.g., enrofloxacin) could be used to assess the detection of mRNA by the RNA-based PCR assay, since this class of antibiotics has affinity for DNA gyrase and/or topoisomerase IV, preventing bacterial DNA replication (Ezelarab et al., 2018). If DNA replication is blocked, transcription does not occur and bacterial mRNA is not produced. The evaluation of the viability PCR assay with bactericidal compounds would be more feasible *in vitro* with cultured *M. hyopneumoniae*, since this type of antibiotics are listed as critically important for humans and animals and are not usually available for use in food-producing animals (Scott et al., 2019; Tang et al., 2017).

Second, another important factor to consider is the concentration of *M. hyopneumoniae* in the animals or the cultures treated with antibiotics and posteriorly subject to DNA or RNA-based PCR testing. In the present study, detection differences between the two PCR assays were assessed in the peak of the acute infection, when the bacterial load is expected to be highest (Arsenakis et al., 2016; Roos et al., 2016; Sørensen et al., 1997). Additionally, pigs in this study were experimentally infected on two consecutive days and were administered a dose greater than the established minimal infectious dose for *M. hyopneumoniae* disease development (Marois et al., 2010). Thus, post-antibiotic differences in detection of exclusively viable *M. hyopneumoniae* versus genetic material from both viable and non-viable cells could have been obscured by a vast preponderance of viable, metabolically active cells in that specific stage of infection. To overcome this limitation, further studies to assess the efficacy of antibiotic treatment based on the detection of viable bacteria should be designed to target the chronic phase of infection, when bacterial load is lower (Pieters et al., 2009; Sørensen et al., 1997), and antibiotics may potentially have greater impact on bacterial control. Also, the inclusion of naturally infected pigs in such studies, which could have replicated the infection dynamics that commonly occurs in typical infections, would have been beneficial to understand the potential differences between the two PCR assays.

An additional explanation for the slight differences observed between the detection of *M. hyopneumoniae* DNA and mRNA in this study could be that *M. hyopneumoniae* cells displayed a certain degree of antibiotic resistance. However, although *M. hyopneumoniae* has been shown to be resistant to several classes of antibiotics, to date, no resistance phenomena have been described for tetracyclines or pleuromutilins (Gautier-Bouchardon, 2020; Maes et al., 2020). Nonetheless, the presence of subpopulations of bacterial cells that display increased tolerance to antimicrobial killing through mechanisms such as dormancy, and reduced metabolism and ATP levels has been described for bacterial species other than *M. hyopneumoniae* (Lewis, 2007). Additionally, slow-dividing subpopulations of bacterial cells, known as persister cells, that have an increased ability to survive exposure to certain antibiotic concentrations (Balaban et al., 2019), have been hypothesized to exist for *M. hyopneumoniae* (Betlach et al., 2021), although they have not been described to date. Furthermore, another mechanism of pathogen avoidance, such as biofilm formation, may be involved in both immune evasion and antibiotic persistence. For *M. hyopneumoniae*, biofilm formation has been described in abiotic surfaces, in porcine epithelial cells monolayers, and in the lung (Raymond, Jenkins, et al., 2018;

Tassew et al., 2017), and has been associated to increased survivability after exposure to antibiotic concentrations tenfold higher than the described minimum inhibitory concentration (Tassew et al., 2017). Further research aiming at better understanding the bacterial-antimicrobial interactions are needed, and the RNA-based PCR assay used in this study could help shed light in that regard.

In conclusion, this study investigated the bacterial viability of *M. hyopneumoniae* in experimentally infected pigs in the acute phase of infection, both pre- and post-antibiotic treatment. Quantitative differences between the detection of total or viable-only *M. hyopneumoniae* cells, via DNA- or RNA-based PCR, respectively, were observed, suggesting that DNA-based PCR assays may overrepresent the population of viable bacteria. The magnitude of the differences was increased post-treatment, potentially implying that the use of diagnostic methods that allow the relative quantification of viable cells may be more suitable to determine the efficacy of antibiotic treatments than DNA-based methods. However, the clinical and disease control implications of the observed differences are currently unknown and should be investigated in further research efforts.

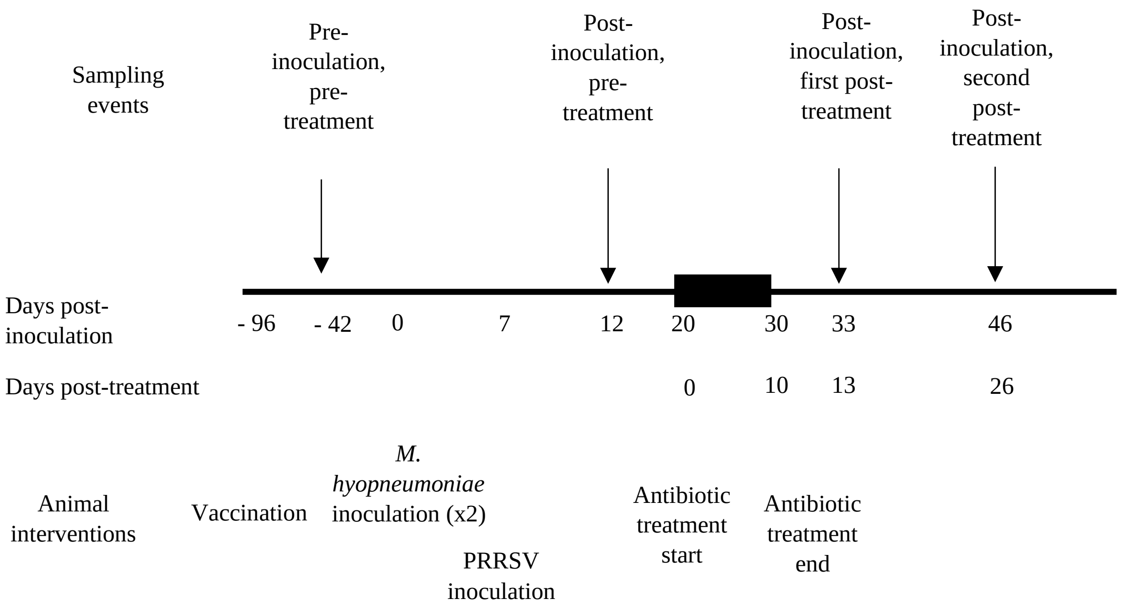

Figure 5.1. Experimental design.

Sampling events: A) Pre-inoculation/pre-treatment (-42 dpi); B) Post-inoculation/pre-treatment (12 dpi/-8 dpt); C) Post-inoculation/first post-treatment (33dpi/13dpt); D) Post-inoculation/second post-treatment (46 dpi/26dpt).

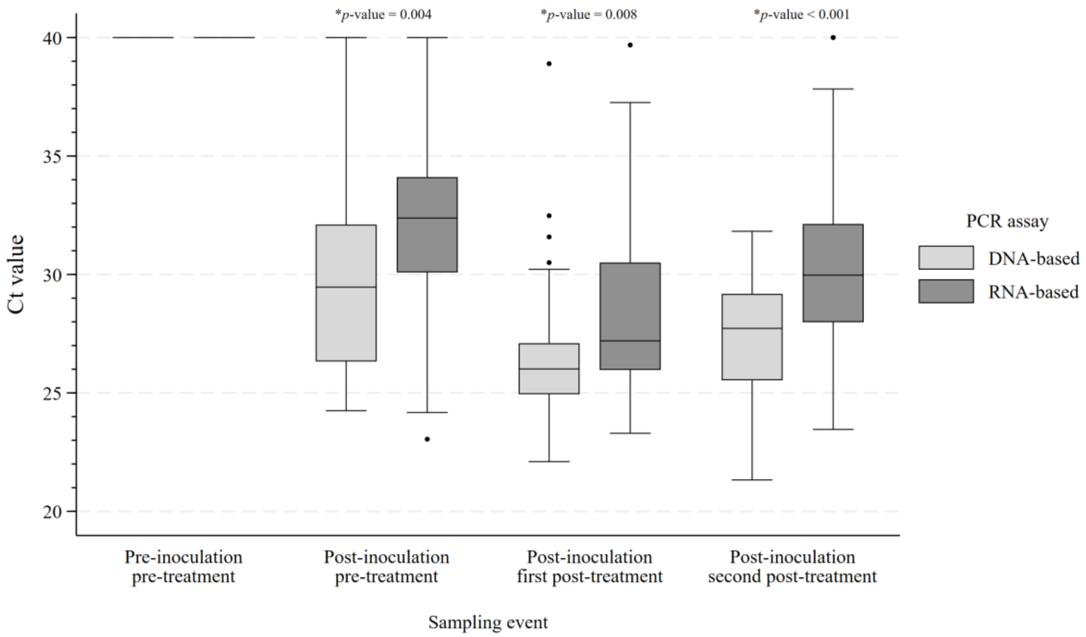

Figure 5.2 Detection of viable (RNA-based) and total (DNA-based) *M. hyopneumoniae* in experimentally infected and treated pigs in commercial conditions. P-values represent differences in mean Ct value between PCR assays witin each sampling event.

A		DNA-based PCR	
		+	-
RNA-based PCR	+	0	0
	-	0	40

B		DNA-based PCR	
		+	-
RNA-based PCR	+	37	0
	-	1	2

C		DNA-based PCR	
		+	-
RNA-based PCR	+	40	0
	-	0	0

D		DNA-based PCR	
		+	-
RNA-based PCR	+	38	0
	-	2	0

Figure 5.3 Frequencies of the categorical results obtained by DNA-based PCR (columns) and RNA-based PCR (rows).

Sampling events: A) Pre-inoculation/pre-treatment (-42 dpi); B) Post-inoculation/pre-treatment (12 dpi/-8 dpt); C) Post-inoculation/first post-treatment (33dpi/13dpt); D) Post-inoculation/second post-treatment (46 dpi/26dpt). There were no significant differences in marginal homogeneity between the two PCR assays using the McNemar's chi-squared test (A) and C), p-value = NA; B) p-value = 1; D) p-value = 0.48

General discussion and conclusions

Diagnosis is a fundamental concept in animal and human health, as it is the foundation for defining clinical intervention and for evidence-based patient or population care. Diagnosis is traditionally defined as the art or act of identifying a disease from its signs in the case of animals, or symptoms in the case of humans. Although the term is inherently used in the medical field, it is increasingly used to define an investigation or analysis of the cause of a problem in other contexts. Examples of the extensive popularity of the term include situations such as when urbanism technicians emit their diagnosis of the traffic problems in morning commutes, or even when auto mechanics provide clients with a multipoint diagnosis of their cars, even if clients just wanted a mere oil change.

In animal infectious diseases, an accurate diagnosis is usually reached following a transdisciplinary approach. This includes, for example, the observation of the clinical picture by a clinician, the detection of the pathogen by a microbiologist, the description of lesions by a pathologist or the measurement of the serological responses of the host by an immunologist. All of these disciplines are subject to constant change and improvement, thanks to advancements in laboratory methodologies, knowledge gained from experimental research, or extrapolation of information obtained in other diseases or animal species. However, it is important to realize that the detection of a pathogen or the observation of signs, by themselves, do not equal diagnosis of disease, as this usually requires objective interpretation of combined factual data into a whole entity. Thus, the results obtained by the different disciplines are critically judged and interpreted by the person that emits the diagnosis, the diagnostician.

Since the first identification of *M. hyopneumoniae* in 1965, several advancements in microbiological techniques and research have contributed to an improved ability to diagnose different aspects of the disease caused by this pathogen. However, there are still remarkable knowledge gaps and opportunity areas in *M. hyopneumoniae* diagnostics. In particular, the determination of bacterial viability status has been a recurrent diagnostic limitation. Distinguishing live from dead bacteria can seem obvious and simple, but it can have important implications for understanding the pathogenesis, epidemiology and control of the diseases caused by *M. hyopneumoniae*. Another aspect of diagnostic ambiguity for *M. hyopneumoniae* is related to the interpretation of its detection by PCR. As previously stated, a correct interpretation of the results obtained by laboratory techniques, such as PCR testing, is critical to guide interventions in the field. However, certain challenges exist when the origin of the detected nucleic acids is uncertain, or when the persistence of detection of the nucleic acids in different conditions is unknown. Therefore, the main goal of this dissertation was to address some of the current limitations and advance the knowledge of *M. hyopneumoniae* diagnosis.

The tissue tropism of *M. hyopneumoniae* is considered to be restricted to the respiratory tract, specifically to the air conductive system. However, extra-pulmonary localization of the bacterium has been described in experimental infection studies, both in terms of bacterial isolation and PCR detection. Additionally, research directed at understanding how *M. hyopneumoniae* cells can traffic outside the respiratory tract has been generated *in vitro*, and although some mechanisms of cellular spread have been suggested, to date, they have not been proved *in vivo*. Therefore, the detection of *M. hyopneumoniae* by PCR in processing fluids (PF) in infected sow herds was unprecedented and could not be explained with the current knowledge on the pathogenesis of the disease. Indeed, detection was obtained simultaneously to a clinical respiratory outbreak in a previously negative sow farm, perhaps suggesting that the finding in PF could be biologically relevant and thus, potentially convenient for diagnostic purposes. Chapter 2 of this thesis dissertation aimed at expanding the scope of the investigation on the detection of *M. hyopneumoniae* in PF by including various farms with different statuses for the disease, from various production systems, and by regularly sampling for an entire year at each. Additionally, the possible origin of the detected genetic material was also explored via sequencing analysis. From this study, it was evident that the detection of *M. hyopneumoniae* genetic material by PCR in PF might be a common finding rather than a sporadic event, as there was consistent detection across positive farms of different production systems, regardless of sow parity or season of the year. Moreover, this study identified environmental contamination with *M. hyopneumoniae* vaccine products as the main potential origin or cause for detection in PF, with the hypothesis that genetic material was mechanically vectorized in the hands and clothes of farm workers, that usually vaccinate weaning pigs and then move to other rooms to process younger pigs.

The repercussions of the results observed in chapter 2 can be radically summarized in a question: “what does a positive PCR result really mean?” Of course, it implies the amplification of a sequence of nucleic acid specific to a pathogen, but the context is essential for understanding the true meaning of the result. Is the result representing an active infection or colonization, or just the residual remnants of uncleared nucleic acids in the milieu where the sample is collected?

For *M. hyopneumoniae*, there is limited information available to answer the abovementioned questions and thus, efforts at characterizing the persistence of detection of *M. hyopneumoniae* DNA by PCR in different situations are crucial for the correct interpretation of diagnostic results and consequently for defining the efficacy of control and elimination strategies. This is especially relevant for this pathogen since PCR detection in a clinical specimen is generally interpreted as that the animal from which the sample was collected is infected.

It has been well established that DNA molecules are extremely stable in the environment and resistant to degradation even in extreme conditions. However, the persistence of detection of *M. hyopneumoniae* DNA in defined, measurable conditions had not been investigated and was addressed in chapters 3 and 4 of this thesis dissertation. In chapter 3, the persistence of detection of *M. hyopneumoniae* DNA from non-viable cells was investigated *in vivo* through an experimental infection study involving the introduction of autoclave-inactivated bacteria in the trachea of pigs and subsequent sampling. Moreover, in chapter 4, the persistence of DNA detection was assessed *in vitro* by applying extreme inactivating conditions to *M. hyopneumoniae* cultures and monitoring the detection levels by PCR over time. Combined, the results indicated that, while DNA detection of non-viable *M. hyopneumoniae* extensively persisted *in vitro*, in the closed environment of the culture tubes, the same did not occur *in vivo*, as DNA was not detectable by PCR in the respiratory or the lymphatic systems, not even shortly after inoculation.

The knowledge gathered from the aforementioned studies has important implications for the diagnosis of *M. hyopneumoniae*. First, the protracted persistence of the detection of DNA in the environment, even after autoclaving, may raise awareness of the potential for sample contamination, whether from the environment or from other samples (i.e., cross-contamination). This can undoubtably be an important problem for *M. hyopneumoniae* DNA testing and subsequent result interpretation. Thus, studies to identify sources and minimize the impacts of sample cross-contamination in the field are needed, and could be applied to develop protocols and define best practices for sample collection. Another field application for which this information can be used is to refine internal biosecurity protocols that would address not only the transmission of *M. hyopneumoniae* between different groups of pigs, but also the interference that the carry-over of genetic material can have on the reliability of molecular diagnostic data.

Second, even though detection of non-viable *M. hyopneumoniae* did not occur *in vivo*, limitations arising from the experimental setting were faced in the study, such as number of pigs, housing conditions and health status, that are intrinsically different from a field-based setting. Thus, research that investigates the drivers of nucleic acid persistence in a more realistic, commercial farm setting is needed to generate broadly applicable results.

Finally, it appeared clear that *M. hyopneumoniae* can be detected in the environment by PCR long after being inactivated and thus, there is no relationship between PCR detection and bacterial viability. Although perhaps obvious, this has important implications for the diagnosis of *M. hyopneumoniae* and thus, methods to determine bacterial viability were needed. Such methods could be used to better understand the nature and the origin of detected *M. hyopneumoniae* in certain epidemiologic scenarios.

Chapter 4 of this thesis dissertation focused on developing a PCR-based assay for the exclusive detection of viable *M. hyopneumoniae* by targeting its mRNA. This viability PCR assay was applied to *M. hyopneumoniae* cultures of known viability status and compared to a real-time PCR assay targeting the DNA of the bacterium. The newly developed assay detected only viable or very recently inactivated bacteria, showing its ability to differentiate between viable and non-viable cells, while all cells were consistently detected by DNA-based PCR irrespective of the viability status of the cultures. Additionally, changes in transcriptional levels were observed throughout different stages of bacterial growth, suggesting that the viability assay can be also used to monitor the growth dynamics of *M. hyopneumoniae in vitro*.

Based on the findings of the study, the developed diagnostic assay can be directly applied in different field scenarios for which determining the viability of *M. hyopneumoniae* has been a limitation. For example, monitoring bacterial clearance post-infection at late stages of the chronic infection can be challenging, as bacterial shedding is commonly investigated via DNA-based PCR and transmission models. Both instances are not ideal, as PCR detects both viable and non-viable cells and thus, it does not provide information about potential infectiousness of the detected *M. hyopneumoniae*. Also, the use of naïve animals to serve as sentinels in transmission models can oppose the 3Rs of animal research, and replacement of this method with another one that does not imply the use of research subjects, such as the viability PCR assay, can be beneficial.

Another example of the potential applicability of the viability PCR assay is to evaluate bacterial clearance post-antibiotic treatment, as a way to infer efficacy of antibiotic treatments against *M. hyopneumoniae*. Insight on the latter was gained in chapter 5, where the viability PCR and the DNA-based PCR were compared in parallel to characterize the bacterial clearance post-treatment with two different bacteriostatic antibiotics. Findings from this study showed differences between the detection of *M. hyopneumoniae* DNA and mRNA at different timepoints, suggesting that DNA-based PCR assays may overrepresent the population of viable bacteria. The magnitude of the differences was increased post-treatment, potentially implying that the use of diagnostic methods that allow the relative quantification of viable cells may be more suitable to determine the efficacy of antibiotic treatments than DNA-based methods. However, this study targeted the acute phase of *M. hyopneumoniae* infection when bacterial load is higher, probably obscuring differences in the clearance of viable cells that could be more evident in chronic stages of disease. Also, the mode of action of bacteriostatic antibiotics could hinder the discriminative properties of the viability PCR, since mRNA can be consistently produced by bacterial cells even if protein synthesis is inhibited. Nevertheless, the clinical and disease control implications of the observed differences are currently unknown and should be investigated in further research efforts. Therefore, chapter 5 highlighted promising field applicability of the viability PCR assay, although further research and study design refinements should follow the initial first steps derived from this field-based study.

Taken together, the work generated in this thesis dissertation addressed several challenges surrounding the appropriate interpretation of diagnostic data of *M. hyopneumoniae* in different epidemiologic scenarios. The essential information provided can be applied to enhance and optimize control and elimination efforts for this pathogen.

Based on the knowledge obtained from this thesis dissertation, and especially from the tools developed and evaluated in it, forthcoming efforts should focus on several research areas. Different aspects of the epidemiology of the disease would benefit from studies addressing bacterial viability, including:

- Evaluation of the effect that disinfectants and cleaning products used in farm operations have on the removal of residual nucleic acid from *M. hyopneumoniae* from environmental surfaces and especially from sampling materials used for *ante-mortem* sample collection. Similarly, the effect that disinfecting compounds have on the viability of different bacteria has been obtained and incorporated in their commercial labels. However, killing efficacy data has not been obtained specifically for *M. hyopneumoniae* and is needed. Combined, this information can be used to design and improve protocols of sample collection aiming at preventing environmental and cross-contamination of samples, and can be included in internal biosecurity protocols.
- Assessment of the potential for indirect transmission of *M. hyopneumoniae*. Knowledge on the viability of *M. hyopneumoniae* in different materials present in swine farms is limited and perhaps has lacked sensitivity since it is based on bacterial isolation. The viability PCR assay developed in this dissertation could

help identify materials or conditions favorable for *M. hyopneumoniae* viability and generate insights on the risk of indirect transmission of the pathogen through fomites or surfaces.

- Use of the viability PCR assay for quality control of the lung inocula used for controlled exposure of pigs to *M. hyopneumoniae* in disease control and eradication strategies.
- Inclusion of the viability PCR assay in the monitoring of bacterial shedding post-infection, especially at late stages of the chronic infection. Increased persistence of *M. hyopneumoniae* detection by PCR post-infection, surpassing the previously established 240 days, has been recently reported. Insights on the origin and the viability of the detected genetic material should be obtained, as well as information on the potential factors that drive extended persistence.

Advanced knowledge on the conceptual microbiology and pathogenesis of *M. hyopneumoniae* could be obtained from studies incorporating viability determination and comprising:

- Further evaluation of the viability PCR assay using other bacterial inactivation methods, including the use of different classes of antibiotics, hydrogen peroxide, U.V light irradiation or sonication, among others.
- Further evaluation and comparison of the growth dynamics of different *M. hyopneumoniae* strains by investigating their transcriptional activity *in vitro* using the developed viability PCR. Differences in growth kinetics could potentially

indicate differences in virulence and thus, strains could be selected based on these characteristics to be included in vaccine development studies.

Finally, new insights on disease control based on antibiotic treatment could be gained from studies encompassing:

- Determination of the potential of the viability PCR assay to serve as a measurement of bacterial growth in antimicrobial susceptibility testing based on minimum inhibitory concentration (MIC) analysis of multiple antibiotic compounds for *M. hyopneumoniae*.

The delineation between life and death is complex and debatable, and several considerations need to be included in the meaning of cell viability in microbiology. However, the importance of determining bacterial viability in the context of *M. hyopneumoniae* disease diagnostics is evident and has been addressed in this thesis dissertation. The field application of the generated information, as well as the future research that will follow will have a significant impact in the *M. hyopneumoniae* control and elimination and consequently, will be highly beneficial for the welfare of pigs and for the wellbeing of swine workers, producers, and veterinarians.

An unattributed Latin proverb states: "*bona diagnosis, bona curatio". *The affirmation, modernly translated as "good diagnosis, good cure", is a fundamental concept of all forms of medicine, in that accuracy in disease diagnosis is a pre-requisite to their optimal control. This thesis dissertation has modestly contributed to a better diagnosis of *M. hyopneumoniae* and will aid present and future swine diagnosticians.

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