Epidemiology of *Mycoplasma hyorhinis* in U.S swine production systems

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Maria Jose Clavijo Michelangeli

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Albert Rovira, Co-advisor
Dr. Robert Morrison, Co-advisor

August 2014

ACKNOWLEDGMENTS

I am very thankful for all of the people who have provided invaluable support, time and guidance during this amazing journey. The work presented here was only possible because of such people and I have no words to express my gratitude.

I was truly fortunate to have such a supportive committee. I thank them for their invaluable contributions to my professional development and I know that we will continue to strengthen our professional relationships over time.

I will always be grateful for my advisor, Dr. Albert Rovira. Thank you for not hesitating to be my advisor when changes occurred, for always teaching me to be critical, for the many insightful discussions and the constant challenge. I really appreciate you giving me the flexibility to pursue my scientific interests and for always respecting my opinions. I have learned the most from you and I look forward to future scientific collaborations.

I thank my co-advisor, Dr. Bob Morrison. Thank you for accepting me as your student. For always asking the most simple, yet most challenging and thought provoking questions. You have taught me to think out of the box and to approach situations in a critical manner.

I thank Dr. Michael Murtaugh, committee chair. I thank you as well for accepting to be part of my committee. You are continuously encouraging students to be the best they can. You were one of the biggest challenges during my program that undoubtedly positively shaped my scientific and personal development. Thank you for always being available for me, for sharing your ideas and for giving me valuable feedback and advice.

I would like to thank Dr. Jeff Zimmerman for being part of my committee. Thank you for your positive criticism, support and patience throughout my program. I truly appreciate you always taking the time to make it to UMN for our meetings. It has been a true honor to have such a reputable scientist and a good friend in my committee.

The opportunity to come to Minnesota was only possible because of Dr. Simone Oliveira. She is the one who believed in me and offered me a position. She gave me the wonderful opportunity to be part of the swine group for two consecutive summers, prior to graduating from Veterinary school. I thank you for being such an amazing mentor.

I would like to thank my extended “committee members” and mentors, Drs. Peter Davies, Srinand Sreevatsan, Tim Johnson, Randall Singer and Scott Dee. Their support, ideas, feedback and knowledge have been instrumental during my program and I am sure that without their guidance my program would have not been the same.

I thank the faculty members of the UMN swine group: Drs. Montserrat Torremorell, Tom Molitor, Han Soo Joo, Maria Pieters, Marie Culhane, Jim Collins, Kurt Rossow, Andres Perez and John Deen, for sharing their immense knowledge, for the productive discussions and exposure to different areas related to swine health and production.

I am very grateful for the swine practitioners and producers that participated in our studies and helped promote *M. hyorhinis* research. I truly appreciate the time and help they invested into our projects. A special thanks to Dr. Deborah Murray and the New Fashion Pork team, Dr. Laura Brunner and the Holden Farms group, Dr. Gill Paterson, Dr. Tim Loula, Dr. Paul Yeske and the rest of the SVC team and Dr. Steve Olson and the Dennis Magnusson group.

I would like to thank former and current VDL staff and researchers for their support over these 4 years and for sharing their valuable knowledge with me. I have learned a lot from each one of them. A special thanks to: Annie, Alyssa, Brian, Gayle, Jason, John, Katie, Karen, Mark, Michelle, Rodney and Yin.

I wish to thank Lisa Hubinger. Her support and help was truly appreciated and cherished.

I would also like to acknowledge all of the funding agencies for their support and for making these studies possible: National Pork Board, Zoetis Animal Health (Dr. Everett Rosey), University of Minnesota-Emerging and diseases signature program and the UMN Swine disease eradication Center.

I thank Ethan Spronk, Andrea Ladinig, Megan Thompson and Megan Bandrick for assisting with sample collection in different studies. Your help was very valuable to me.

My time in the program would never have been the same without the support and fun moments spent with my fellow swine graduate students and researchers. I will never forget all of the insightful scientific debates (and not so scientific!), farm trips and fun lunch gatherings at the student center. I really enjoyed getting to know each one of you and I truly appreciate all of the help you guys provided in all of my studies. I am sure our friendships will be long lasting. A very special thanks to: Ana, Andres, Carmen, Cesar, Dane, Daniel, Doug, Fabio, Jisun, Leticia, Lucas, Matt, My, Nitipong, Nubia, Steve, Seth, Susan, Tim and Victor.

I would like to thank the best and sweetest friends a person could ever have: Andre, Anto, Caro, Dani, Greys and Maite. Thank you for all of those times you made me smile. I miss you!

I thank the most important people in my life, my family. It was their endless support, love and encouragement that gave me the strength to go through this journey. To complete a

PhD you certainly need a team and they were my team:

I thank my unofficial godmother and aunt Mary, my comadre, Maria Luisa and my cousin, Meme, for all of their love and for making me laugh even as we are a thousand miles apart.

My wonderful parents and role models, Claret and Jose, who instilled in me the love of science and always encouraged me to go on every adventure (especially this one!). I admire them for all of their accomplishments in life, for giving me all they ever had and for teaching me by example and not advice. I am proud to call you both my parents.

I will never forget when I was a child, finishing a swimming race, I looked up and there was my brother giving me the biggest smile and a hand to hold on to. That love and that support have never changed. My brother, has the biggest heart and is certainly the best padrino I know. Thank you, Pepi.

I thank the sweetest and kindest person on earth, the love of my life, my rock, Jean Paul. Thank you for your everyday encouragement and love, for making my days happy ones, for your constant patience and for giving me the greatest joy in my life, Tomas.

DEDICATION

To the people that make my days bright: Claret, Jose, Pepe, Jean Paul and Tomas.

TABLE OF CONTENTS

LIST OF TABLES

LIST FIGURES

GENERAL INTRODUCTION

Mycoplasma hyorhinis, first described in 1955, belongs to the mollicutes class, which comprises more than 100 mycoplasma species that infect humans and animals (Razin et al., 1992). This wall-less bacterium is considered a commonly occurring microorganism in swine, where it attaches to the ciliated upper respiratory tract (Switzer. 1955; Friss. 1971). Through mechanisms that are still not understood, *M. hyorhinis* can invade systemic organs causing mainly fibrinous polyserositis and arthritis, but it has also been implicated as a cause of pneumonia and otitis (Kobisch and Friss, 1996). *M. hyorhinis* has recently emerged as one of the main concerns of swine veterinarians dealing with post-weaning morbidity and mortality (Leuwerke. 2009; Murray D. 2012). Since the introduction of PCR in 2009, *M. hyorhinis* is detected in about 50% of polyserositis and 12% of arthritis cases submitted to the Minnesota Veterinary Diagnostic Laboratory (MVDL) every year. In the field, swine practitioners are applying different antibiotic and autogenous vaccine protocols with varying degrees of success (Murray. 2014). Part of the minimal success is due to the lack of essential epidemiological information, which is scarce and obsolete. The answers to basic epidemiological questions such as "when are pigs becoming infected?", "how quickly does the infection spread?" or "what are the sources of infection?" are unknown. The main goal of this dissertation was to advance the knowledge on the infection dynamics and the epidemiology of *M. hyorhinis* in swine populations in the US. Such information will contribute to the better implementation of control and prevention strategies for this pathogen.

To begin to generate the epidemiological data there is a need for highly sensitive and specific diagnostics tools that can track the infection in live animals. Several diagnostic methods have been used for the detection of *M. hyorhinis*. Isolation of this pathogen requires special media and growth can take up to 14 days (Hayflick and Chanock, 1965). More recently, a number of PCR protocols targeting *M. hyorhinis* have been described (Kobayashi et al., 1996; Timenetsky et al., 2006; Tocqueville et al., 2014). However, most of them are based on traditional end-point PCR protocols and therefore are not capable of quantifying the amount of bacterial DNA in a sample. Furthermore, the performance of these PCR protocols has not been evaluated with a wide range of clinical samples. The lack of accurate and thoroughly evaluated diagnostic tools represents a major limitation to study the dynamics of *M. hyorhinis* infection in swine populations. Chapter 2 describes the development and evaluation of a highly sensitive and specific quantitative PCR (qPCR) assay for the detection and quantification of *M. hyorhinis* in field samples.

Little is known about the epidemiology of this pathogen. Most available studies were carried out on slaughter pigs or on pigs submitted to diagnostic laboratories (Friis and Feenstra, 1994). Essential information, such as the prevalence of pigs in commercial populations is absent. Moreover, the limited information available was generated from studies carried out in the 60's and 70's and since then the swine industry has gone through dramatic changes that may have affected the ecology of pathogens such as *M.*

hyorhinis. In Chapter 3, the prevalence of *M. hyorhinis* infection was estimated in different age groups across three commercial swine populations by means of a cross-sectional study.

Information related to *M. hyorhinis* transmission and spread within infected populations is largely unknown. Specifically, the relationship between sow and piglet infection, as well as the transmission between pigs pre- and post-weaning has not been studied yet. To accomplish this, a longitudinal sampling of pigs over time is necessary to accurately characterize the temporal patterns of *M. hyorhinis* infection and to identify the possible sources of infection for young pigs. In Chapter 4, the prevalence and incidence of *M. hyorhinis* in naturally infected pigs and the relationship between sow infection and serological status with piglet infection was evaluated through a longitudinal study.

Finally, another largely unknown aspect related to the epidemiology of *M. hyorhinis* is the characterization of the genetic profiles of *M. hyorhinis* isolates circulating in swine production systems. Differences in strains within and between swine populations could impact the observed dynamics of infection and spread. Antigenic differences between *M. hyorhinis* isolates have been shown by seroreactivity to specific antisera (Friis, 1971). Several experimental challenge studies have demonstrated differences in virulence *in vivo* (Shulman et al., 1970; Gois and Kuksa, 1974). In Chapter 5, a sequence based tool, multilocus sequence typing (MLST) assay, was developed

based on housekeeping and surface encoding proteins to differentiate *M. hyorhinis* strains, to assist local epidemiological or outbreak investigations, design future control strategies as well a serve as a potential tool to study the evolutionary biology of this species.

CHAPTER I
LITERATURE REVIEW

Sections of this chapter have been published in:

Rovira A, Clavijo MJ, Oliveira S. 2010. *Mycoplasma hyorhinis* infection of pigs. Acta Sci Vet 38 (Supl 1):s9-s15.

1.1 Biology of the genus Mycoplasma highlighting *M. hyorhinis*

Described for the first time more than 100 years ago, *Mycoplasmas* (class *Mollicutes*) represent a unique group of bacteria, characterized by their minimal genome size (600 to 1200 kb), lack of a cell wall and low G+C content (20-40%). These bacteria have evolved from A+T rich, walled gram-positive bacteria and are considered a fast evolving group based on recent phylogenetic analyses (Ciccarelli et al., 2006). Because such quick evolution has lead not only to the loss of a cell wall but also to a reduced coding capacity and loss of biosynthetic pathways, it has been described as a degenerative evolution (Citti and Blanchard, 2013). In fact, Mycoplasmas represent the smallest and simplest self-replicating organisms known to man (Baseman and Tully, 1997). Currently the *Mollicutes* class comprises close to 200 species.

1.1.1 Phylogeny and taxonomy

The emergence of Mycoplasmas is considered a fairly recent event, based on the evolutionary scale, occurring about 600 million years ago from the *Streptococcus* phylogenetic branch (Maniloff. 1996). They belong to the Mollicutes class, alongside 4 other genera; *Acholeplasma, Anaeroplasma, Spiroplasma* and *Ureaplasma.* Advancements in the areas of phylogeny and taxonomy within the class have been primarily accomplished by 16S rRNA analysis, phenotypic characteristics and serology. However, in some cases the phylogenetic information is not in accordance with the observed taxonomical classification. For instance, the phylogenetic tree based on the 16S

rRNA places *M. mycoides* more closely related to the Spiroplasmas than with the *Mycoplasmas* (Dybvig and Voelker, 1996). With the advent of whole genome sequencing, more *Mollicutes* species are being sequenced; hence information generated will improve phylogenetic analysis and consequently allow for accurate taxonomic classification. Recently, phylogenetic studies based on 179 genes common between 32 mycoplasmas revealed that *M. hyopneumoniae* was closely related to *M. flocculare*. *M. hyorhinis* was found to be part of the same clade as *M. conjunctivae, M. flocculare and M. hyopneumoniae* (Siqueira et al., 2013). Nearly identical phylogenetic relationships have been seen using the gene 16S rRNA (Liu et al., 2012). A pairwise alignment between *M. hyorhinis* HUB-1 whole genome and other related mycoplasmas, revealed a 99% identity with other *M. hyorhinis* genomes, 81% with *M. hyopneumoniae*, 74% with *M. conjunctivae* and 73% with *M. suis* (Figure 1).

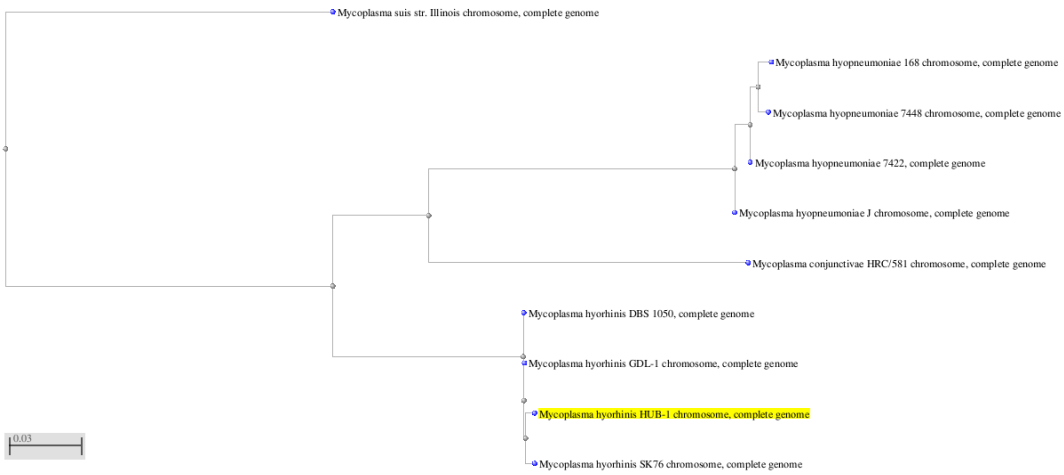


Figure 1. Dendrogram created using the Neighbor Joining tree method on pairwise nucleotide alignment between *M. hyorhinis* HUB-1, DBS 1050, GDL-1 and SK76 genomes, *M. suis*, and 4 *M. hyopneumoniae* genomes. Alignment was created using the tBLASTn algorithm and tree was created with the BLAST tree view option.

1.1.2 Ecology and habitats

Mollicutes have been found infecting a wide range of hosts comprising, plants, arthropods, reptiles, birds, mammals and humans (Razin S and Hayflick, 2010; Whitcomb et al., 1989). Within the *Mycoplasma* genus more than 100 species, have been described (Razin et al., 1998). These organisms are normally found within the mucous membranes of the respiratory and urogenital tracts, serosal surfaces, joints and the eyes. Mycoplasmas typically present a strict host and tissue specificity. However, exceptions have been seen in *M. hyorhinis,* which can infect the upper respiratory tract as well as serosal surfaces of pigs. There is evidence supporting *M. hyorhinis* infection of tissues

from different hosts, such as human gastric tissues (Yang et al., 2010). Additionally, *M. hyorhinis* is a frequently occurring cell-culture contaminant, together with *M. orale* and *M. argininis*, providing even more evidence of survivability within multiple habitats.

Various mycoplasma species such as *M. fermentans*, *M. hominis*, *M. genitalium* and *M. penetrans* have shown the ability to enter epithelial cells, instead of the common location on the cell surface; (Lo et al., 1998). Although *M. hyorhinis* adheres to the cell surface, a recent study showed the invasion of a *M. hyorhinis* strain into melanoma cells in vitro. This mechanism may increase survivability of the pathogen by avoiding the host immune system and antibiotics (Kornspan et al., 2010).

1.1.3 In vitro culture

The degenerative evolution that has occurred in mycoplasmas has led to a complete lack of essential *de novo* synthesis pathways, as they adjusted to a parasitic life. In fact, it has been shown that mycoplasmas lack all genes related to amino acid synthesis and almost all related to biosynthesis of vitamins and fatty acids (Fraser et al., 1995; Himmelreich et al., 1996). Therefore, in order to survive, mycoplasmas need to obtain these from the host or artificial culture medium. The media is usually based on beef heart infusion, peptone, yeast extract and serum with various supplements (Hayflick. 1965). *M. hyorhinis* measures 0.2-0.3 μm. On solid media, the organism grows down in the agar and some areas grow over the central embedded area giving rise to colonies with the appearance of

"fried eggs" (Hayflick. 1965). A subset of *M. hyorhinis* isolates are not able to grow in special media due to inhibitors found in the media, some of which are components of peptones and yeast extract.

1.1.4 Genome structure, organization and characteristics

Five *M. hyorhinis* strains have been fully sequenced and the ATCC 17981 strain is in progress. The HUB-1 strain originated from china and was isolated from the respiratory tract of pigs (Liu et al., 2010). Strain SK76 was obtained from the joint of a pig with arthritis (Goodison et al., 2013). Strains MCLD, GDL-1 and DBS 1050 all originated from cell cultures. MCLD was derived from a human melanoma cell line (Kornspan et al., 2011). GDL-1 was obtained from a tissue culture and the DBS 1050 strain was an isolate that did not grow on conventional media (Calcutt et al., 2012; Dabrazhynetskaya et al., 2014). The completed genomes consisted of a circular chromosome of approximately 838kb with an average G+C content of about 26%. The number of protein-encoding genes ranged from 654 to 741 with an average size of about 364 amino acids. The genomes contain one copy of each 5S, 16S, and 23S rRNA genes, which are not organized in an operon. A unique feature of *M. hyorhinis* is that they possess a set of variable lipoproteins (*vlp*) that undergo high-frequency surface antigenic variation, which allows them to evade the host immune system. Strains can contain up to 7 different *vlp* genes (defined as A through G). HUB-1 and SK76 strains contain all 7 genes organized in the following order 5'-*vlpD*-*vlpE*-*vlpF*-insertion sequence-*vlpG*-*vlpA*-IS-*vlpB*-*vlpC*-3'.

DBS 1050 and GDL-1 strains both lack the *vlpG* gene and the MCLD strain contains only 4 *vlp* genes (*vlpB, C,* and *E*) with no insertion sequence within the locus (Kornspan et al., 2011; Calcutt et al., 2012; Dabrazhynetskaya et al., 2014).

Besides the reduced biosynthetic pathways, mycoplasmas carry a limited set of genes related to DNA replication, transcription and translation. A single DNA polymerase has been detected in *M. hyorhinis* which lacks the 3'-5' proofreading exonuclease activity, which might contribute to high mutation rates observed in mollicutes (Woese. 1987) Mycoplasmas also have a reduced number of genes related to DNA repair. Furthermore, some carry a defective uracil-DNA glycosylase (*ung*), which could contribute to the low G+C content observed. Some mycoplasmas such as *M. gallisepticum* and *M. capricolum* do not carry this gene. (Williams and Pollack, 1990). DNA-dependent RNA polymerase detected in mycoplasmas is similar to those detected in other bacteria. Regarding translation, M. hyorhinis carries 30 tRNAs and 21 tRNA synthetases. Due to their A+T rich genomes, codon usage favors codons with A and T, usually in the third position (Dybvig and Voelker, 1996). Finally, the UGA codon within mycoplasmas translates to the amino acid tryptophan instead of a stop codon.

In spite of their minimal genome, these organisms are able to survive on the host and evade its defense system and also produce the metabolic machinery needed for cell growth. One mechanism that allows them to achieve this with such small genomes is

chromosomal gene rearrangement. Three types of recombination occur in mycoplasmas: homologous recombination through RecA-dependent pathways; site-specific recombination through lysogenic mycoplasma viruses, transposable elements and site-specific inversions; and illegitimate recombination (Bork et al., 1995).

1.1.5 Pathogenesis

The molecular mechanisms of mycoplasma pathogenicity are not completely understood. Most mycoplasmas are considered commensals and hardly provoke the death of its host, thus disease is mostly chronic in nature. Research suggests that most of the damage to the host could be due to an exaggerated immune response, instead of a direct effect of components of the bacterium (Razin et al., 1998). Nonetheless, several bacterium-mediated mechanisms have been proposed; (1) oxidative stress induced by the generation of hydrogen peroxide and superoxide radicals, (2) host cell maintenance and function disorders produced by reduction of nutrients due to the parasitic effect of mycoplasmas, (3) surface structures that increase mycoplasma adhesion and provide immunoregulatory properties (4) high surface plasticity through phase and antigenic variation which allows mycoplasmas to evade host immune response, (5) production of enzymes such as ATPases, hemolysis, nucleases, and proteases introduced into the host cells causing tissue damage and chromosomal abnormalities, and (6) ability to go intracellular which evades host immune response and antibiotic effect (Rosengarten and Wise, 1990; Razin. 1991; Sugimura et al., 1992; Hahn et al., 1998).

One striking feature observed in mycoplasmas are the rapid and reversible genetic changes (phase and antigenic variation) observed in a group of genes that produce great surface variation. As described earlier, *M. hyorhinis* has a cluster of genes that encode lipoproteins (*vlp*). Each *vlp* gene represents a single transcription unit that has a poly-A tract in the promoter region. When this tract is 17bp long the vlp is transcribed and translated (Yogev et al., 1991; Yogev et al., 1995). However, any insertion of deletion to this sequence and transcription does not occur. This type of ON/OFF switch of each lipoprotein is obtained through a genetic mechanism termed DNA slippage and results in a non coordinated expression of multiple or a single lipoprotein (Rosengarten and Wise, 1990). Such genetic mechanism not only determines expression of lipoproteins, but also dictates protein size variation by DNA slippage in short direct repeats (Citti et al., 2000; Citti and Baranowski, 2010).

Lastly, the induction of host inflammatory and immune responses by mycoplasmas could modulate cancer progression. There is an ongoing debate regarding the role of *M. hyorhinis* in human cancer since it has been detected in many types of cancer, including gastric and prostate cancer (Huang et al., 2001). Still, a clear causal relationship has not been proven.

1.2 *Mycoplasma hyorhinis* infection in pigs

1.2.1 Etiology

M. hyorhinis was the first mycoplasma isolated from swine (Switzer WP, 1953). It was thought to be the cause of enzootic pneumonia, which was then correctly attributed to *M. hyopneumoniae* (Mare and Switzer, 1965: Goodwin et al., 1965). Isolation of *Mycoplasma hyorhinis* requires special media but can be accomplished relatively easily (Hayflick and Chanock, 1965; Gois and Kuksa, 1974). Colonies can be observed 2-14 days after inoculation of solid media measuring 0.5 mm to 1mm in diameter (Kobisch and Friis, 1996).

Research suggests that *M. hyorhinis* is genetically and antigenically a diverse organism. Antigenic differences between different *M. hyorhinis* isolates have been shown by seroreactivity to specific antisera (Friis. 1971). This antigenic variation might be determined in part by the presence of a highly complex system of vlp expression, which allows for great surface variation (Rosengarten et al., 1991). In addition to this antigenic variation, several experimental challenge studies have shown differences in virulence *in vivo* (Shulman et al., 1970; Gois et al., 1971; Gois. and Kuksa, 1974; Kinne et al., 1991). At the genome level, the use of pulsed-field gel electrophoresis (PFGE) to index variation within the *M. hyorhinis* species has revealed genetic heterogeneity within the species (Barlev and Borchsenius, 1991). Multilocus sequence typing, based on sequencing housekeeping gene fragments, has been recently applied to *M. hyorhinis* and

genetic variation was observed. Within 33 field isolates of *M. hyorhinis* originating from pigs in different regions of France 29 distinct sequence types were observed. (Tocqueville et al., 2014).

1.2.2 Epidemiology

M. hyorhinis is a common inhabitant of the respiratory tract of pigs that, under certain conditions can cause severe systemic disease. Isolation of *M. hyorhinis* associated with clinical disease has been reported in most swine producing countries of Europe, North America and Asia. However, because most studies have been done in slaughter pigs or in pigs submitted to diagnostic laboratories, there is little known about the epidemiology of *M. hyorhinis* in swine farms. It is assumed that piglets get infected from the sows or from older pigs and most of them do not develop disease (Friis and Feenstra 1994; Kobish and Friis, 1996; Thacker. 1996). In one study *M. hyorhinis* was isolated from nasal secretions of 2/27 and from frontal sinus secretions of 1/27 euthanized sows of 1 to 3 years of age (Ross and Spear, 1973). Friis & Feenstra (1994) studied clinical cases with typical lesions of serositis in 3 to 7-wek-old pigs submitted to a diagnostic laboratory in Denmark (Friis and Feenstra, 1994). *M. hyorhinis* was isolated from 82% of lungs, 35% of serosal surfaces, 27% of brains, 25 % of tonsils and 20% of joints of pigs with serositis. In a small sample of pigs with no respiratory or serosal lesions, *M. hyorhinis* was isolated from 87% of lungs, 37% of tonsils and 25% of brains but not from serosal surfaces or joints. In multiple studies *M. hyorhinis* was isolated from the respiratory tract

of both healthy and diseased pigs. Typically, the rate of isolation was higher in diseased pigs than in healthy pigs. In normal pigs, the rate of isolation tended to be higher in studies that used younger pigs, compared to those using slaughter pigs. In another study with Danish pigs, *M. hyorhinis* was isolated from the tympanic cavities of 80% of pigs with otitis media and 52% of pigs with no lesions (Friis et al., 2002). Schulman et al. (1970) isolated *M. hyorhinis* from the nasal cavity of 49% of pigs with rhinitis submitted to a diagnostic laboratory in Finland, while 27% of pigs with no lesions of rhinitis were also positive (Shulman et al., 1970). Similarly, they found 49% of lungs with pneumonia and 17% of normal lungs to be positive. In a different study performed on slaughtered pigs in Norway, 37% of pneumonic lungs were positive for *M. hyorhinis* while only 6% of normal lungs were positive (Falk et al., 1991). In other studies the rate of *M. hyorhinis* isolation from pneumonic lungs was 62% (Friis. 1971; Meyling. 1971) and 74% (Assunção et al., 2005). More recent studies have used PCR to detect *M.* hyorhinis in field samples with similar results. Caron et al. (2000) tested lungs from Canadian slaughter pigs for *M. hyorhinis* by PCR and found 13% of pneumonic lungs positive and only 2% of normal lungs positive (Caron et al., 2000). In a recent study in Germany, 80% of pneumonic lungs and 37% of normal lungs from slaughter pigs were positive for *M. hyorhinis* by PCR (Palzer et al., 2008).

Information regarding the prevalence and timing of infection at the herd level is scarce. In one study, *M. hyorhinis* was isolated from the nasal cavity of 56/61 grower pigs

(30kgs) in Czechoslovakia (Gois et al., 1968). Moreover, most studies related to *M. hyorhinis* infection in pigs were carried out more than 30 years ago. Since then, the swine industry has undergone drastic changes in pig flow and management, thus potentially affecting the ecology of this organism.

1.2.3 Clinical signs and lesions

Most infections are limited to the upper respiratory tract and sometimes the lung and are subclinical. Experimentally, intranasal inoculation with *M. hyorhinis* generally results in subclinical infection (Friis. 1971; Gois and Kuksa, 1974; Lin et al., 2006;Morita et al., 1999). However, when *M. hyorhinis* becomes systemic it can cause severe disease consisting of pleuritis, pericarditis, peritonitis and arthritis, mainly in 3 to 10-week-old pigs. Clinical signs vary depending on the serosal surfaces affected and include fever, dyspnea, swollen joints, lameness, reluctance to move and unthriftiness. Lameness can become chronic and last up to six months. The polyserositis consists of fibrinous or fibrinopurulent pleuritis, pericarditis and/or peritonitis. If the pig survives, lesions progress to chronic serositis with formation of adhesions (Duncan and Ross, 1973; Roberts et al., 1963).

The arthritis is characterized by hypertrophy and hyperemia of the synovial membrane and lymphocyte infiltration with a serosanguinolent exudate, sometimes containing fibrin (Barthel et al., 1972). In chronic arthritis, pannus formation and articular

erosion have been described (Kobish and Friis, 1996). Polyserositis and arthritis have been reproduced experimentally by intraperitoneal inoculation (Barthel et al., 1972; Duncan and Ross, 1973; Friis and Feenstra, 1994; Gois and Kuksa, 1974; Gois et al., 1971; L'Ecuyer and Boulanger, 1970; Magnusson et al., 1998; Roberts et al., 1963; Switzer. 1955) and, in some cases, by intranasal inoculation of *M. hyorhinis* (Gois and Kuksa, 1974; Gois et al., 1971; Kobish and Friis, 1996).

In addition to polyserositis and arthritis, *M. hyorhinis* has been associated with a number of clinical presentations including rhinitis, pneumonia, otitis and conjunctivitis (Morita et al., 1993; Morita et al., 1999; Schulman et al., 1970; Thacker. 1996). However, the significance of *M. hyorhinis* in these disease presentations is unclear. It is important to remember that *M. hyorhinis* is a ubiquitous organism that grows easily in culture media and therefore isolation of *M. hyorhinis* from a diseased animal does not imply causality. In addition, only mild, self-limiting forms of rhinitis, otitis and pneumonia have been reproduced experimentally by inoculation of *M. hyorhinis.* The role of *M. hyorhinis* in swine pneumonia has been a question of debate for decades (Gois et al., 1968; Lin et al., 2006; Taylor-Robinson. 1975). As described in the previous section, *M. hyorhinis* is isolated more frequently in pneumonic than in normal lungs. However, experimental inoculation of pigs with *M. hyorhinis* rarely produces pneumonia and, when it does, it is mild and only observed in a small percentage of the infected pigs (Friis.

1971, Kinne et al., 1991; Lin et al., 2006). Currently, *M. hyorhinis* is considered a secondary invader of pneumonic lesions.

1.2.4 Pathogenesis

M. hyorhinis has been detected by immunofluorescence and/or immunohistochemistry on the ciliary area of the epithelium in nasal cavity, auditory canal, trachea and bronchi of asymptomatic pigs (Kinne et al., 1991; Morita et al., 1993). In pigs with polyserositis, *M. hyorhinis* antigen has been reported within pleural, pericardial and synovial lesions (L'Ecuyer and Boulanger, 1970; Gois et al., 1971; Potgieter and Ross, 1972). In pigs with pneumonia, *M. hyorhinis* was detected coating the bronchiolar epithelium and in the bronchial and alveolar exudate (Meyling. 1971; Gois et al., 1971). However, the mechanisms that allow *M. hyorhinis* to colonize the respiratory tract and to become systemic and cause disease are not well understood. It seems that pathogen factors, host factors and environmental factors all play a role. There are no known virulence factors for *M. hyorhinis.* However, differences in virulence between isolates have been demonstrated in experimental inoculation studies (Gois and Kuska, 1974; Lin et al., 2006; Ross and Switzer, 1963).

The host genetics also seem to play a role in the severity and the presentation of disease. Magnusson *et al.* (1998) inoculated two lines of pigs selected for high and low immune response intraperitoneally with *M. hyorhinis.* They found that pigs selected for

high immune response tended to develop arthritis while in pigs selected for low immune response the disease presented as polyserositis (Magnusson et al., 1998). In a different study, pigs appeared to become more resistant with age (Gois and Kuska, 1974).

Environmental factors such as coinfections with other pathogens or stressful events are probably an important contributor to expression of disease. Kinne et al. (1991) reported an effect of stress on the development of pneumonia after inoculation with *M. hyorhinis* (Kinne et al., 1991). Several studies reported an increased rate of *M. hyorhinis* isolation from pigs coinfected with PRRSV (Kawashima et al., 1996; Kixmöller et al., 2008; Kobayashi et al., 1996; Shimizu et al., 1994) PCV2 (Kawashima et al., 2007) or *Bordetella bronchiseptica* (Gois et al., 1977) and suggested a synergistic effect of these coinfections.

1.2.5 Diagnosis

Immunohistochemistry and immunofluorescence assays have been used for detection of *M. hyorhinis* in tissues (L'Ecuyer and Boulanger, 1970; Gois et al., 1971; Potgieter and Ross, 1972). Binder *et al.* (1989) reported that immunofluorescence was less sensitive than culture for lung samples (Binder et al., 1989). A number of PCR protocols targeting *M. hyorhinis* have been described (Stemke et al., 1994; Kobayashi et al., 1996; Caron et al., 2000; Lin et al., 2006; Timenetsky et al., 2006; Kang et al., 2012; Tocqueville et al., 2014). However, most of them are based on traditional end-point PCR protocols and

therefore are not capable of quantifying the amount of bacterial DNA in a sample. Furthermore, the performance of these PCR protocols has not been evaluated with a wide range of clinical samples and some of them were developed for the detection of *M. hyorhinis* in cell cultures only (Timenetsky et al., 2006). Antibody targeted assays have also been described in the literature (Roberts et al., 1963b; Ross and Switzer, 1963; Gois et al., 1972; Duncan and Ross, 1973; Binder et al., 1989; Friis and Feenstra, 1994; Kawashima et al., 1996). Serological tests have also been developed and an antibody response has been observed in pigs 1 to 2 weeks after inoculation (Duncan and Ross et al., 1973; Gois et al., 1972; Roberts et al., 1963). However, these assays have been employed for research purposes, they are not commercially available and some require euthanasia of the animal.

Attempts to correlate disease with *M. hyorhinis* are complicated due to the commensal nature of this pathogen. Therefore, a full necropsy workup is recommended. Pigs demonstrating lameness, difficulty breathing, or fever should be selected for sampling. Ideally pigs should be in the acute stage and without previous antibiotic treatment. Special care should be taken to ensure that the samples are being aseptically collected from the targeted joints or serosal surfaces of interest for PCR and/or culture. Additionally, specimens of the joint and/or tissue being affected can also be placed into 10% formalin to enable histopathology evaluation. The diagnostic specimens being submitted for isolation and/or PCR testing must be refrigerated and quickly sent to the

diagnostic laboratory for analysis. In arthritis cases, sending the whole leg to the diagnostician might improve the detection of the agent (Gomes-Neto. 2012).

1.2.6 Treatment and control

Because mycoplasmas lack a cell wall, they are naturally resistant to certain antibiotics such as penicillins and cephalosporins. However, several *in vitro* antimicrobial studies have shown that *M. hyorhinis* is susceptible to multiple antibiotics. In one study where the minimal inhibitory concentration of 51 antimicrobial agents was evaluated by serial broth dilution, *M. hyorhinis* was susceptible to lincomycin, clindamycin, furaltadona, kanamycin and all sulfonamides. In contrast *M. hyorhinis* was found to be resistant to all cephalosporines and penicillins (Williams. 1978). Other *in vitro* studies tested 18 different antibiotics by broth and agar dilution and found that this pathogen was highly susceptible to oxytetracyclin, lincomycin and tylosyn, however it was also demonstrated that *M. hyorhinis* was highly resistant to erythromycin (Ter et al., 1991; Wu et al., 2000; Kobayashi et al., 2005). Recent studies have shown that macrolide-resistant *M. hyorhinis* strains quadrupled in the last decade in Japan, allegedly due to multiple use of antimicrobial agents as chemotherapy (Kobayashi et al., 2005). Unfortunately, there are no published reports of the efficacy of these antibiotics under field conditions. It has been reported that antibiotic treatment of *M. hyorhinis* infections is only successful when applied at the early stages of disease (Thacker. 1996) The capacity of *M. hyorhinis* to

invade cells intracellularly has been suggested (Kornspan et al., 2010). Such mechanism could greatly hinder antibiotic treatment efficacy.

CHAPTER II
FIELD EVALUATION OF A QUANTITATIVE PCR ASSAY FOR *MYCOPLASMA HYORHINIS*

Clavijo MJ, Oliveira S, Zimmerman J, Rendahl A, Rovira A. 2014 Field evaluation of a quantitative PCR assay for *Mycoplasma hyorhinis* [Accepted for publication in Journal of Veterinary Diagnostic Investigation]

2.1 Summary

Mycoplasma hyorhinis has recently emerged as an important cause of systemic disease in nursery pigs. However, this bacterium can also be found in the upper respiratory tract of healthy swine. The current study describes the development of a quantitative polymerase chain reaction assay for the detection of *M. hyorhinis* and the evaluation of the assay in both disease diagnosis and disease surveillance using a large number of field samples. The analytical sensitivity was estimated as 12 genome equivalents/µl. The assay was highly specific, detecting all 25 *M. hyorhinis* isolates tested and none of the 19 non-target species tested. Assay repeatability was evaluated by testing different matrices spiked with known amounts of *M. hyorhinis*. Overall, assessment of the repeatability of the assay showed suitable precision within and between runs for all matrices. The coefficient of variation (CV) ranged from 10 to 24%. *Mycoplasma hyorhinis* DNA was detected in 48% of samples (pericardium, pleura, joints, nasal cavity and lungs) from pigs with systemic disease. *Mycoplasma hyorhinis* was detected in nasal (92%) and oropharyngeal swabs (66%), as well as in oral fluids (100%). Potential uses of this tool involve the characterization of the prevalence of this pathogen in swine herds as well as bacterial quantification to evaluate intervention efficacy.

2.2 Introduction

Mycoplasma hyorhinis causes mainly fibrinous polyserositis and arthritis in pigs, and has been implicated as a cause of pneumonia and otitis (Roberts et al., 1963; Gois et al., 1968; Duncan and Ross, 1969; Gois et al., 1971; Gois et al., 1972; Friis et al., 2002; Barthel et al., 2009). This wall-less bacterium is a common finding in the upper respiratory tract of healthy pigs and it is considered to be globally-distributed (Switzer, 1955; Fris. 1971). Stress and concomitant infection with other respiratory pathogens may predispose pigs to *M. hyorhinis* disease (Roberts et al., 1963a; Gois and Kuksa, 1974; Kinne et al., 1991; Magnusson et al., 1998).

Little is known about the epidemiology of this pathogen. The source of infection for young pigs is yet to be elucidated; however, it is presumed that sows can transmit the organism to their offspring and subsequent transmission between pigs follows (Kobisch and Friis. 1996). To advance the knowledge of the dynamics of *M. hyorhinis* infection in affected herds, polymerase chain reaction (PCR) testing could be done on nasal swabs of pigs at the individual level or on oral fluids at the group level. However, because *M. hyorhinis* is a commensal of the upper respiratory tract of pigs, there is a need for a diagnostic tool that not only detects but also quantifies the amount of bacterial DNA in the sample.

Several diagnostic methods have been used for the detection of *M. hyorhinis*. Isolation of this pathogen requires special media and can take up to 14 days to grow (Hayflick and Chanock, 1965; Gois and Kuksa, 1974). Immunohistochemistry and immunofluorescence assays have been used for detection of *M. hyorhinis* in tissues (L'Ecuyer and Boulanger, 1970; Gois et al., 1971; Potgieter and Ross, 1972). Antibody targeted assays have also been described in the literature (Roberts et al., 1963b; Ross and Switzer, 1963; Gois et al., 1972; Duncan and Ross, 1973; Binder et al., 1989; Friis and Feenstra, 1994; Kawashima et al., 1996). However, these assays have been employed for research purposes, they are not commercially available and some require euthanasia of the animal. A number of PCR protocols targeting *M. hyorhinis* have been described (Stemke et al., 1994; Kobayashi et al., 1996; Caron et al., 2000; Lin et al., 2006; Timenetsky et al., 2006; Kang et al., 2012; Tocqueville et al., 2014). However, most of them are based on traditional end-point PCR protocols and therefore are not capable of quantifying the amount of bacterial DNA in a sample. Furthermore, the performance of these PCR protocols has not been evaluated with a wide range of clinical samples and some of them were developed for the detection of *M. hyorhinis* in cell cultures only (Timenetsky et al., 2006).

The current study describes the development and evaluation of a highly sensitive and specific quantitative PCR (qPCR) assay for the detection and quantification of *M. hyorhinis* in field samples. The use of this quantitative assay in epidemiological

investigations will increase our understanding on the dynamics of *M. hyorhinis* infection in swine populations.

2.3 Materials and Methods

2.3.1 *M. hyorhinis* isolates and culture conditions

A reference strain, *M. hyorhinis* ATCC17981D (Strain, 17981D. American Typing Culture Collection, Manassas, VA) was utilized for the development and standardization of the qPCR assay. In addition, 25 *M. hyorhinis* isolates cultured from clinical cases of polyserositis were tested. These isolates were recovered from different serosal surfaces such as pleura, pericardium, peritoneum, and synovial membrane. All *M. hyorhinis* isolates were cultured in modified Hayflick's media incubated aerobically at 37°C for two to 14 days in 3 ml broth tubes and with 5% CO_2 at 37 °C on solid agar media (Lefebvre et al., 1987). Isolates were stored at -80 °C following confirmation of growth on solid media.

2.3.2 *M. hyorhinis* DNA isolation

Genomic DNA from all samples was obtained using a commercial kit according to manufacturer's specifications (DNeasy Blood & Tissue kit, Qiagen, Germantown, MD). The starting material for bacterial isolates was the growth from 4 plates harvested from solid media and re-suspended in 0.5 ml of Dulbecco's phosphate buffered saline (D-PBS)

(Sigma-Aldrich, St. Louis, MO) or 0.5 mL of modified Hayflick's media. The efficiency of the DNA extraction was assumed to be 100%. The final product was stored at -80°C and used as the DNA template.

2.3.3 PCR primers and probe

The partial sequence of 16S small subunit ribosomal RNA gene of *M. hyorhinis* (GenBank GU227402.1) was aligned to other 16S ribosomal RNA partial sequences of closely related bacterial pathogens, including *Mycoplasma hyopneumoniae* (GenBank JN935889, GU227407.1, Y00149.1)*, Mycoplasma flocculare* (GenBank GU227403)*,* and *Mycoplasma hyosynoviae* (GenBank AY973563.1) using ClustalW (Chenna et al., 2003) in MEGA (MEGA 5.0, Tempe, AZ). *Mycoplasma hyorhinis* species-specific regions were selected to design forward and reverse primers, as well as the probe. Selected sequences were evaluated using online software (Primer 3 software, Cambridge, MA). The fluorogenic probe was labeled with FAMTM-dye as reporter at the 5′ end and TAMRATM-dye as quencher at the 3′ end (Taqman®. Life Technologies, Grand Island, NY). The ideal annealing temperature (t_a) was 54 C°.

2.3.4 Quantitative PCR conditions

The qPCR reaction for *M. hyorhinis* was prepared in a volume of 25 µl consisting of 5 µl of the template DNA, 12.5 µl of Master Mix solution (QuantiFast Probe PCR kit. Qiagen, Germantown, MD), 1 µl of ROX dye, 0.125 µl of each 40 µM reverse (MHR- 5'- AAG

TGA AGC TGT GAA GCT C -3') and forward (MHF- 5'-GAT GTA GCA ATA CAT TCA GTA GC-3') primers, 1.25 µl of 5 µM fluorogenic probe (5'-FAM-CGG ATA TAG TTA TTT ATC GCA-TAMRA-3') and 5.0 µl of DNAse and RNAse free H_20. The reactions were carried out on a real-time PCR system (ABI 7500 fast. Life Technologies, Grand Island, NY). The cycling protocol consisted of 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 15 seconds and 54 °C for 50 seconds. All samples were run in duplicates and H_20 was employed as the no target control in all reactions. The negative extraction control was 0.5 ml of D-PBS (Sigma-Aldrich, St Louis, MO). Absolute quantification of *M. hyorhinis* DNA was estimated from the extrapolation of the values from a 10-fold dilution standard curve included in each run.

2.3.5 Analytical sensitivity

Genomic DNA was extracted from the *M. hyorhinis* ATCC reference strain and the amount of DNA was quantified by spectrophotometry (Nanodrop 2000, Wilmington, DA). The number of genome equivalents (geq) was determined based on the genome size of *M. hyorhinis,* 839,615 base pairs (bp**),** approximately equivalent to 1 fg DNA per cell. With the objective of estimating the minimum number of geq that can be detected with the qPCR, 10 and 2-fold dilutions were made on nuclease free water to achieve template concentrations from 10^7 to 10^0 geq/µl of extracted DNA. Results were compared to those obtained by conventional end-point PCR (Timenetsky, et al., 2006).

2.3.6 Analytical specificity

The basic local alignment search tool (BLASTn) was utilized to evaluate sequence similarity of the primers and probe to other targets. Non-specific binding was evaluated by running the newly developed protocol by end-point PCR. The presence of potential products resulting from non-specific binding was assessed by electrophoresis on 1% agarose gels stained with ethidium bromide. In addition, a total of 19 reference strains of bacterial species commonly found in swine were tested with the qPCR: *Actinobacillus equuli, Actinobacillus indolicus, Actinobacillus minor*, *Actinobacillus pleuropneumoniae* (serovar 7)*, Actinobacillus porcinus*, *Actinobacillus rossii*, *Erysipelothrix rhusiopathiae, Haemophilus parasuis*, *Bacillus cereus, Truperella pyogenes, Bordetella bronchiseptica, Escherichia coli, Pasteurella multocida, Streptococcus suis, Salmonella* sp.*, Staphylococcus aureus, Mycoplasma hyopneumoniae, Mycoplasma flocculare* and *Mycoplasma hyosynoviae.*

2.3.7 Repeatability (within-laboratory precision)

Evaluation of assay repeatability was carried out following the guidelines established by the Clinical and Laboratory Standards institute (Clinical and Laboratory Standard Institute, 2004). Spiked pericardium, nasal, oral fluid, and modified Hayflick's medium (control) matrices were utilized to estimate the repeatability of the assay. A total of 20 nasal and pericardial swabs, as well as 10 mL of oral fluids were collected from designated pathogen-free (DPF) pigs. Swabs were pooled with 10 mL of D-PBS (Sigma-

Aldrich, St. Louis, MO). All matrices were *M. hyorhinis* negative by qPCR and end-point PCR prior to the spiking. Matrices were spiked with a concentrated *M. hyorhinis* culture to achieve a high and a low bacterial concentration (10^6 and 10^3 geq per µl of extracted DNA). All samples used in the validation of the assay were extracted twice; at the beginning of the experiment and 5 days later following the protocol described above. Samples were run in triplicates, twice per day at different times, for 10 consecutive days. Each run employed a negative and positive extraction control as well as a PCR negative control (H_2O).

Assay repeatability was evaluated by performing graphical analysis, estimating coefficient of variation values (CV), and by utilizing various mixed models to assess sources of variation. The CV values were estimated by utilizing the equations reported in the CLSI guidelines for "Device or Laboratory precision", with the modification to increase the number of replicates from 2 to 3. In addition, data was log transformed, and various mixed models were fit to estimate both the mean response at each combination of matrix and dose and the variability at each of one of these levels. To explore how the variances differed between combinations, models were fit to the cumulative data and then to each dose/matrix combination and each dose/matrix/extraction combination. The full model included concentration, matrix, extraction, and all interactions as main effects, with random effects for day, run, and triplicate. The models for each dose/matrix combination had "extraction" as main effect and "day" and "run" as random effects; the

models for dose/matrix/extraction had “day” and “run” as random effects. All calculations were performed using a statistical software (3.0.2, Ime4 package 2013, R Core Team, San Francisco, CA).

2.3.8 Field samples

2.3.8.1 Postmortem samples from suspect herds

To compare the diagnostic performance of the qPCR with a previously published end-point PCR (Timenetsky, et al. 2006), a total of 50 clinical samples from pleura (14), peritoneum (3), pericardium (8), joint (18), nasal cavity (3) and lung (3) submitted to the Minnesota Veterinary Diagnostic Laboratory were tested. These samples originated from cases suggestive of *M. hyorhinis* evidencing fever, dyspnea and reluctance to move. Discordant results between the two PCR protocols were fully investigated by reviewing pathological findings, and by sequencing the PCR product.

2.3.8.2 Antemortem samples from known positive herds

A total of 70 nasal swabs, 30 oropharyngeal swabs and 34 oral fluids samples from 8-week-old pigs, were tested for the presence of the bacteria by qPCR. These pigs originated from herds with clinical disease associated with *M. hyorhinis* infection.

2.3.8.3 Antemortem and postmortem samples from a negative herd

With the goal of testing a negative population, 24 pericardium and 10 nasal swabs were tested for *M. hyorhinis* by qPCR only. These samples were obtained from adult pigs originating from a high biosecurity, DPF herd.

All samples were extracted following the procedure described above, with the exception that the starting material differed. For postmortem samples, the starting material was 200 µl of a solution resulting from vortexing a sample of fibrin from the affected serosal surface re-suspended in 3 ml of PBS. For samples from live pigs (nasal and oropharyngeal swabs) and from designated pathogen-free (DPF) pigs (pericardial and nasal swabs), swabs were suspended into 0.5 ml of PBS, followed by vortexing and centrifugation. The remaining pellet was used for DNA extraction.

2.3.9 Sequencing

A subset of PCR products obtained from testing clinical samples and lower dilutions from the analytic sensitivity assay were purified using a commercial kit (QIAquick PCR purification kit, Qiagen, Germantown, MD). Samples were sequenced in both directions with the primers described above to obtain double coverage using a 96 capillary sequencer (ABI 3730xl BigDye Terminator v 3.1. Life Technologies, Grand Island, NY). Obtained sequences were entered in BLAST for target confirmation.

2.4 Results

2.4.1 Analytical sensitivity and specificity

The analytical sensitivity of the *M. hyorhinis* qPCR was determined with a series of 10 and 2-fold dilutions of the ATCC strain. The standard curve of *M. hyorhinis* DNA displayed linearity over 7 log units, as exemplified by a $r^2 > 0.99$ for 10 consecutive set ups. The undiluted initial DNA concentration measured by spectrophotometry was 23 ng/µl, which is equivalent to approximately 2.3 x 10^7 geq/µl. The detection limit was determined to be 12 geq/µl of extracted DNA (4.8 x10^3 geq per mL of culture broth), which corresponded to a CT value of 34.2. The estimated qPCR efficiency was 91.29%. The length of the qPCR product estimated using an ethidium bromide-stained agarose gel was consistent with the expected length of 151 bp (data not shown). PCR products from positive samples (lower dilutions) were sequenced. These sequences were analyzed by basic alignment using BLAST and were homologous to the *M. hyorhinis* 16S rRNA gene. The detection limit of the conventional end-point PCR was the same as the qPCR.

Introduction of the primer and probe sequences in BLASTn did not reveal highly similar sequences other than *M. hyorhinis*. There was no evidence of non-specific binding in a 1% ethidium bromide-stained agarose gel. The qPCR was highly specific, detecting exclusively *M. hyorhinis* isolates. All 19 non-*M. hyorhinis* bacterial isolates

tested negative. In contrast, all 25 *M. hyorhinis* isolates from clinical cases tested positive.

2.4.2 Repeatability (Within-laboratory precision)

The repeatability within and between runs and days appeared to be high and stable across all matrices (Fig. 1). There was a noticeable effect of the extraction on the estimated bacterial concentration, as evidenced by differences between the results obtained during the first 5 days (first extraction) compared to the last 5 days (second extraction) and compared to the other matrices, oral fluids showed lower precision overall (Fig. 1). Considering the differences observed between the first and second extraction, CV estimates were calculated independently for each combination of matrix, dose and extraction. The CVs ranged from 10 to 13% in the high concentration sample with the exception of the oral fluid matrix, extraction 1, which had a CV of 24%. CVs ranged from 13-21% for the low concentration sample (Fig. 2).

Mixed models revealed 3 sources of variation, namely, “day”, “run within day”, and “replicate” (residual) (Fig. 3). Within the low-dose, “run” and “replicate” accounted for most of the variation, whereas in the high-dose all 3 sources contributed to the observed variation.

2.4.3 Field samples

The qPCR detected *M. hyorhinis* in 48% (24/50) of the samples from clinically affected pigs, compared to 34% (17/50) using the conventional end-point gel based PCR. The 7 samples with discordant results tested positive by qPCR and negative by gel based PCR. Samples with discordant results had a low bacterial load (average 10^2 geq/µl) compared to the rest of positive samples (average 10^4 geq/µl) and originated from multiple sampling sites (pleura, pericardium, joint and nasal cavity). The PCR products of these samples were sequenced and reliable DNA sequences were obtained for 6 of them. Sequence analysis of the discordant results confirmed the samples as *M. hyorhinis*, with 100% similarity.

Ninety-two percent (65/70) of nasal swabs, 66% (20/30) of oropharyngeal swabs and 100% (34/34) of oral fluids samples of pigs originating from clinically affected herds tested positive for *M. hyorhinis* by qPCR. The average bacterial concentration of these samples was 10^5 geq/µl. All 24 pericardial swabs and 10 nasal swabs originating from adult DPF pigs were negative for *M. hyorhinis*.

2.5 Discussion

The goal of the current study was to develop a highly sensitive and specific qPCR for *M.*

hyorhinis and to evaluate its application for diagnosis and surveillance with a wide range of sample types. The assay was shown to be highly sensitive, detecting 12 geq/µl of extracted DNA. While the analytical sensitivity of a previously described end-point PCR (Timenetsky et al., 2006) and the qPCR were identical, the results of a side-by-side comparison performed on 50 clinical samples indicated a higher sensitivity of the qPCR. The specificity of the assay was demonstrated by the lack of positives in a large panel of swine bacteria and by negative results obtained from clinical samples expected to be negative.

Assay repeatability was evaluated by testing different matrices spiked with known amounts of *M. hyorhinis* DNA. Different types of matrices were used to mimic diagnostic samples commonly tested in a diagnostic setting. Overall, assessment of the repeatability of the assay showed suitable precision within and between runs. Mixed models revealed various sources of variation (day, run and replicate) with the majority of the variation due to run-within-day. The estimated coefficient of variation ranged from 10 to 24%. Similar values have been obtained with quantitative PCR assays targeting *Mycoplasma genitalium* and *Mycoplasma haemocanis* (Svenstrup et al., 2005; Barker et al., 2009). There was an impact of less than one log of the extraction batch on the estimated amount of *M. hyorhinis* detected. This was an unexpected finding since sample extraction was performed both times following the exact same protocol. This study, however, was not designed to evaluate the effect of extraction on qPCR results. The impact of extraction on assay reproducibility warrants further study. A slight decrease in the expected bacterial

load observed with the oral fluids matrix might be due to overall decreased extraction efficiency due to the sample composition or to the presence of PCR inhibitors (Das et al., 2009).

Previously published end-point PCR assays were developed for the detection of *M. hyorhinis* in cell culture (Timenetsky et al., 2006), in lung samples (Kobayashi et al., 1996; Stemke et al., 2004) or in formalin-fixed-paraffin embedded tissues from pigs with polyserositis (Kang et al., 2012). Recently, a qPCR was developed with a reported detection limit of 125 geq/µl. However, the use of the assay was only assessed using bacterial isolates and not clinical samples. Furthermore, assay repeatability was not evaluated (Tocqueville et al., 2014). In the current study, *M. hyorhinis* DNA was detected in multiple sample types originating from clinically (pleura, pericardium, peritoneum, joint, nasal and bronchial swabs) and non-clinically affected pigs (oral fluids, oropharyngeal and nasal swabs). The proportion of positive samples from pigs with disease (pleura, pericardium, peritoneum, nasal cavity and lung) observed in the current study (48%) is in accordance with that observed in other reports. In one study, *M. hyorhinis* was isolated from 35% of serosal surfaces of pigs submitted to a diagnostic laboratory (Friis and Feenstra, 1994). Approximately 55% of serosal tissues from affected pigs submitted to the Minnesota Veterinary Diagnostic Laboratory were positive by end-point PCR (Rovira et al., 2010). The high proportion of qPCR positive oral fluids, nasal and oropharyngeal swabs from clinically healthy pigs was expected since *M.*

hyorhinis is a commensal organism of the upper respiratory tract of pigs. The detection of *M. hyorhinis* in oral fluids has not been assessed before. While an in-depth validation of this sampling technique needs to be carried out for detection of *M. hyorhinis*, the practicality and non-invasiveness nature combined with the ability to perform herd level surveillance makes this technique promising (Prickett et al., 2008). The lack of detection of *M. hyorhinis* in pericardial and nasal swabs from DPF pigs was expected since these were clinically healthy adult pigs with no lesions at necropsy and originating from negative herds. In addition to the origin of the DPF pigs, the non-detection of *M. hyorhinis* in nasal swabs could be due to as-yet-unidentified host factors. For example, it is possible that older pigs are able to clear *M. hyorhinis* infection more efficiently than younger pigs.

In summary a rapid, high-throughput, sensitive and specific assay for detection of *M. hyorhinis* in multiple sample types is described. The results of this study demonstrate the potential of the assay for accurate detection of *M. hyorhinis* in field samples. Furthermore, the assay shows a potential to be used to characterize the nasal colonization dynamics of this pathogen in different group ages and to quantify the bacterial load to evaluate treatment efficacy.

Figure 2.1 Panel of plots showing the bacterial load, expressed as genome equivalents per µl, measured by the quantitative polymerase chain reaction assay, of four matrices spiked with two different *M. hyorhinis* bacterial concentrations. Two extractions were performed. Samples from each set of extractions were tested for 5 days. Two plots are provided for each matrix based on two concentration levels – H = High level and L = Low level. Circles (A) represent the replicates for the first run and crosses (B) represent replicates for the second run.

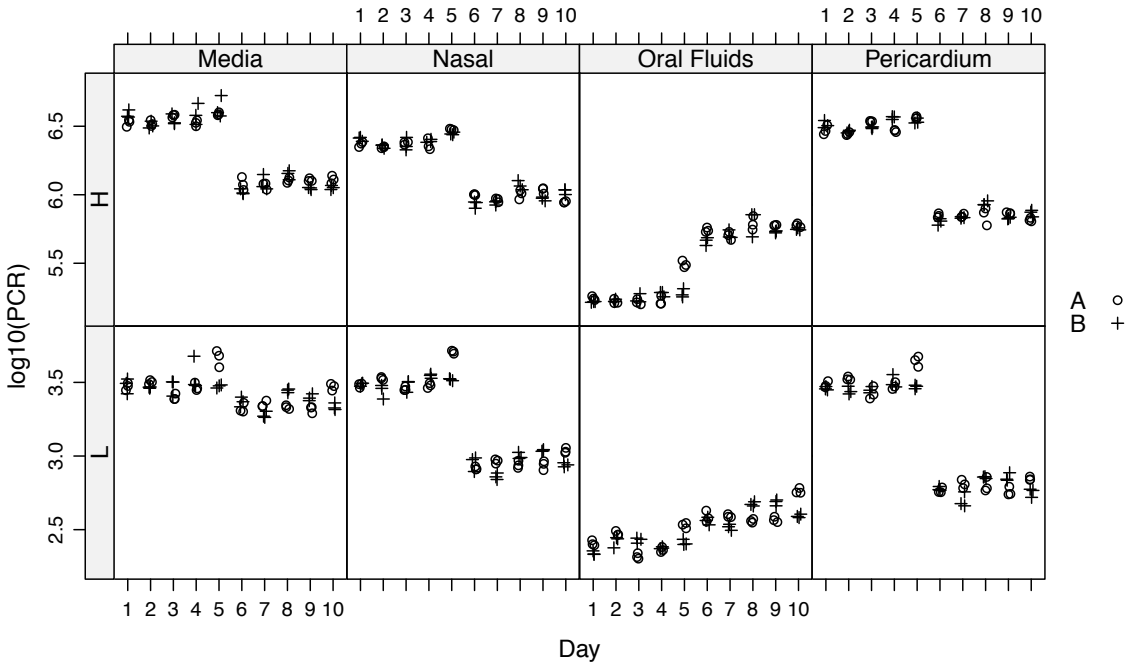


Figure 2.2 Coefficient of variation (CV%) estimates for each concentration level and extraction, within each matrix.

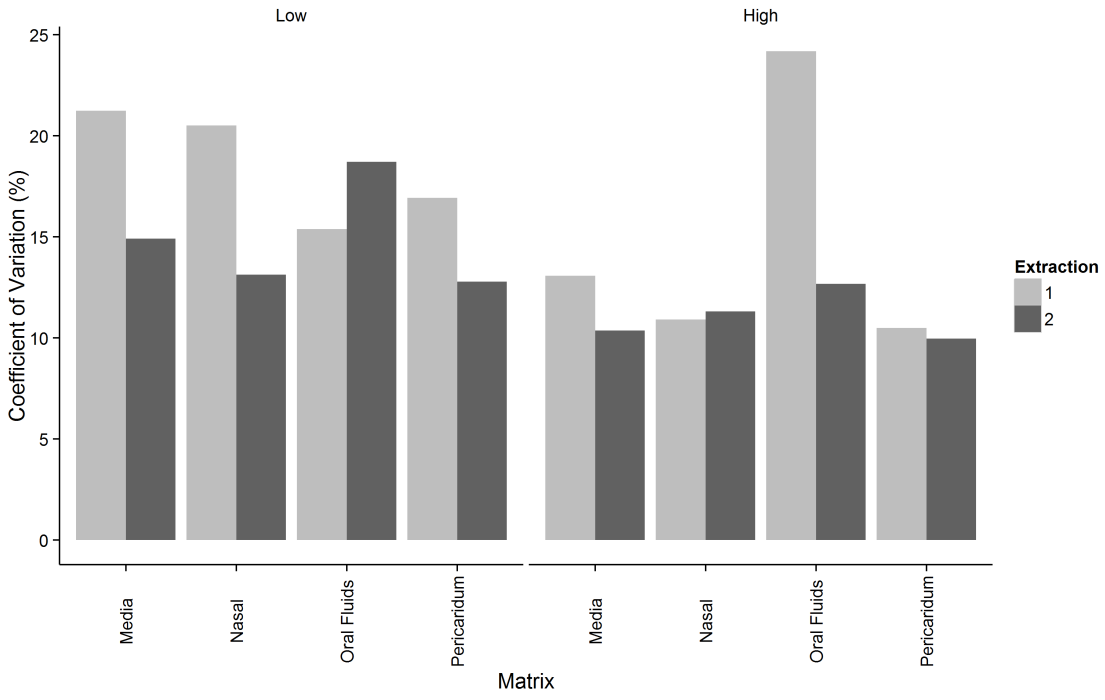
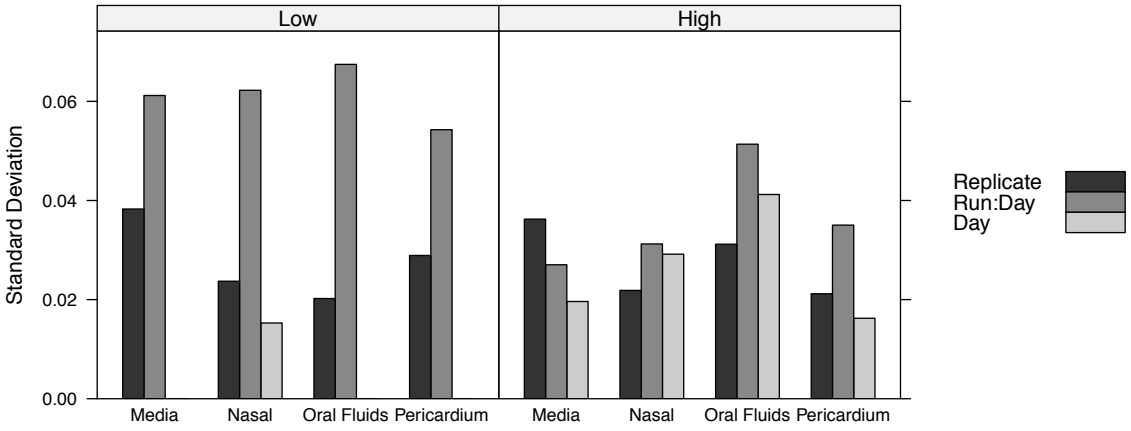


Figure 2.3 Standard deviation estimates obtained from mixed models for each source of variation.



CHAPTER III
PREVALENCE OF *MYCOPLASMA HYORHINIS* IN THREE COMMERCIAL SWINE POPULATIONS FROM THE U.S.A. MIDWEST

Clavijo, MJ., Oliveira S., Murray D., Rovira, A. Prevalence of Mycoplasma hyorhinis in three commercial swine populations from the U.S.A. Midwest. [in prep, to be submitted to Veterinary Microbiology]

3.1 Summary

M. hyorhinis has recently been recognized as one of the main causes of nursery mortality in U.S. swine herds. However, current epidemiological information on this pathogen is scarce and obsolete. The objective of this study was to estimate the prevalence of *M. hyorhinis* infection in different age groups across three commercial swine populations. In each herd, nasal swabs were collected from 60 sows, 60 piglets in each group of 1, 7, 14 and 21 days of age as well as 30 pigs in each group of 28, 35, 42, 49, 56, 63, 70 and 77 days of age. Oral fluids were also collected from the same post weaning pigs. *M. hyorhinis* was detected in the nasal cavity of 5/60 sows in herd A, 3/60 in herd B and none in herd C. In herd A and B, where clinical cases of *M. hyorhinis* were present, the prevalence of infection was low in pre-weaning piglets (avg=8%) and high in post-weaning pigs (avg=98%). In contrast, in herd C, where *M. hyorhinis* clinical signs were absent, the prevalence pre-weaning and post-weaning was low (1.3% and 3.3%) until the last week in the nursery when prevalence spiked to 100%. A total of 7/8 oral fluids tested PCR positive for *M. hyorhinis* in herds A and B, while 1/8 tested positive in herd C. Ten pigs were necropsied in each herd to assess the presence of *Mycoplasma hyorhinis* clinical disease at the time of sampling. In herds A and B necropsied pigs had polyserositis lesions and *M. hyorhinis* was detected by PCR in such lesions. In herd C *M. hyorhinis* was not detected in any of the necropsied pigs. This is the first report of the prevalence of *M. hyorhinis* in different age categories. In these herds, high prevalence of

M. hyorhinis nasal infection in weaned pigs was associated with the presence of *M. hyorhinis* associated disease and the detection of the agent in polyserositis cases.

3.2 Introduction

First described in 1955, *Mycoplasma hyorhinis* (*M. hyorhinis*) is commonly found in swine herds, where it attaches to the ciliated upper respiratory tract of pigs (Switzer. 1955; Friss. 1971). Through mechanisms that are still not understood, the bacterium can invade systemic organs and cause disease. The two main lesions caused by this pathogen are polyserositis and arthritis, usually in 3 to 10 week old pigs (Gois et al., 1968; Poland et al., 1971; Ross 1992; Friis and Feenstra, 1994). Systemic infection in finishing stage pigs is usually characterized by arthritis; nonetheless, most *M. hyorhinis* infections are generally subclinical (Gomes-Neto et al., 2012). Clinical signs reported include fever, dyspnea, lameness and reluctance to move (Gois et al., 1971; Ross 1992). *M. hyorhinis* has recently emerged as one of the main concerns of swine veterinarians dealing with post-weaning morbidity and mortality (Leuwerke. 2009; Murray. 2012). The rate of detection of *M hyorhinis* by PCR in polyserositis and arthritis cases submitted to the Minnesota Veterinary Diagnostic Laboratory (MVDL) in 2010 revealed that this pathogen was detected in 55% of polyserositis and 12% of all arthritis cases (Rovira et al., 2010). These results highlight the importance of *M. hyorhinis* on post-weaning mortality in the US.

M. hyorhinis is thought to be transmitted from sow to piglet and piglet-to-piglet afterwards (Kobisch and Friis, 1996). However, there is limited information regarding the ecology and epidemiology of this agent. Most epidemiological studies available were

carried out on slaughter pigs or on pigs submitted to diagnostic laboratories (Friis and Feenstra, 1994; Assunção et al., 2005; Palzer et al., 2008). Essential information, such as timing of infection and prevalence of infected pigs in a population is also lacking. Moreover, most of the research took place in the 60's and 70's and the swine industry has gone through dramatic changes since then. Swine herds have evolved from relatively small family-owned farrow-to-finish farms into consolidated systems with much larger and multisite operations. Those changes may have affected the ecology of pathogens such as *M. hyorhinis*. The main objective of this study was to estimate the prevalence of *M. hyorhinis* infection in different age groups across three commercial swine populations.

3.3 Materials and methods

3.3.1 Study design

To determine the prevalence of *M. hyorhinis* infection a cross-sectional nasal sampling was performed. The survey was carried out between July 2010 and October 2010 in three herds. In each herd, a nasal swab sample was collected from randomly selected sows, piglets of 1, 7, 14 and 21 days of age and postweaning pigs at 28, 35, 42, 49, 56, 63, 70 and 77 days of age. All procedures regarding the handling and sampling of pigs were approved by the Minnesota Institutional Animal Care and Use Committee (protocol # 1004A80280).

3.3.2 Herd selection

Sow herds were selected based on the following criteria: (1) herd size of at least 1500 sows, (2) multisite production system, (3) history of *M. hyorhinis* associated disease confirmed by PCR or bacterial culture, (4) no *M. hyorhinis* vaccination, (5) location within driving distance from Saint Paul, MN and (6) willingness of veterinarians and producers to participate.

3.3.3 Sampling protocol

3.3.3.1 Breeding herds

In each herd, sixty nasal swab samples (BBL CultureSwab, Stuart single plastic applicator, Becton, Dickinson and Co., Sparks, MD, USA) were obtained from sows housed in the farrowing room and pigs of 1, 7, 14 and 21 days of age by introducing a nasal swab deep into each nostril. Parity and treatment information was recorded. Parity stratified random sampling was performed for sow selection. Simple randomization was used to select one piglet from each litter of desired age. If the number of litters of a specific age was not sufficient to fulfill the desired sample size of 60, litters of ± 1 day were selected. Because of the lack of data available on true prevalence of *M. hyorhinis* at these ages, a large sample size of 60 animals per sampling age was used. This sample size

would enable estimating a wide range of possible prevalence estimates with acceptable precision. For example, it would allow estimating a prevalence of 0.5 with 95% confidence and an error of +/-13% or a prevalence of 0.1 or 0.9 with 95% confidence and an error of +/-8%. (http://epitools.ausvet.com.au/).

3.3.3.2 Nursery sites

Thirty nasal swabs (BBL CultureSwab, Stuart single plastic applicator, Becton, Dickinson and Co., Sparks, MD, USA) were collected from randomly selected post-weaning pigs in each group at 28, 35, 42, 49, 56, 63, 70 and 77 days of age. A smaller sample size was used in this case because extreme prevalence estimates were expected. A sample size of 30 would allow estimating a prevalence of 0.9 with 95% confidence and error of +/-11% (http://epitools.ausvet.com.au/). In total, 540 nasal swab samples were collected in each post-weaning flow. All swabs collected from sows and pigs were refrigerated following sample

As a side objective, the possibility of detection of *M. hyorhinis* in oral fluids from nursery pigs was explored. One oral fluid sample was collected from a pen with approximately 70 animals at 28, 35, 42, 49, 56, 63, 70 and 77 days of age, for a total of 8 oral fluid samples in each farm. For oral fluid collection, a non-bleached cotton rope was hanged for approximately in one randomly selected pen at the pigs shoulder height. Pigs were allowed to chew on the rope for 20-30 minutes. Following sample collection, the

rope was placed into a sterile plastic bag, the moistened part of the rope was squeezed, and the obtained fluid was poured into 25ml tubes and stored at -20 °C until testing (Prickett et al., 2008).

3.3.4 Clinical and pathological evaluation

With the purpose of confirming the involvement of *M. hyorhinis* in nursery mortality, in each herd, a total of 10 post-weaning pigs with clinical signs suggestive of polyserositis (prostration, cough, lameness, abdominal breathing, and fever of at least 105 °F), as well as 10 clinically healthy pigs of the same cohort were humanly euthanized, necropsied and sampled. The age of the sampled pigs was determined by the herd veterinarian, based on current mortality rates and clinical signs observed at the time of the study. Samples were aseptically collected from lung, heart, spleen, kidney, liver and inguinal lymph node of each pig and immersed in 10% formalin for histological examination. Swabs from serosal surfaces (pleura, pericardium, synovial membrane) were tested for *M. hyorhinis* by quantitative PCR (qPCR), for *Haemophilus parasuis* by PCR and for aerobic bacteria by aerobic culture. Aerobic bacterial culture was also performed from lung and liver. A tissue pool containing lung, spleen and inguinal lymph node was tested for porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) by PCR. Bronchial swabs were tested for *M. hyopneumoniae* and *M. hyorhinis* by PCR and a lung sample was utilized for detection of *Influenza A* virus by PCR.

3.3.5 Sample processing and quantitative PCR protocol

All nasal swabs collected from sows and pigs were stored at -20 °C until testing. At sample processing swabs (nasal, serosal, and bronchial) were resuspended in 0.5 mL of D-PBS (Sigma-Aldrich, St Louis, MO), vortexed and centrifuged. *M. hyorhinis* DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Germantown, MD). All samples were tested by a qPCR assay based on the 16S rRNA gene (Clavijo et al., submitted 2014). Briefly, the qPCR reaction was prepared in a volume of 25 µl consisting of 5 µl of the template DNA, 12.5 µl of Master Mix solution, 1 µl of ROX dye, 0.125 µl of each 40 µM forward (MHF- 5'-GAT GTA GCA ATA CAT TCA GTA GC-3') and reverse (MHR- 5'–AAG TGA AGC TGT GAA GCT C-3') primers, 1.25 µl of 5 µM TaqMan probe® (5'-FAM-CGG ATA TAG TTA TTT ATC CGA-TAMRA-3') and 5.0 µl of DNAse free H_20. Reactions were carried out in the ABI 7500 fast real-time PCR system (Life Technologies, Grand Island, NY) at 95°C for 3 min, 35 cycles of 95°C for 15 sec and 54°C for 50 sec. Bacterial quantification, expressed as genome equivalents/swab (geq/swab), was achieved through extrapolation of the values from a 10-fold dilution standard curve included in each run. Samples were tested in duplicate together with a negative and positive extraction control as well as a PCR negative control (H_2O).\

3.3.6 Statistical analysis

Apparent prevalence was calculated as the number of positives divided by number of pigs tested for each age group, along with its 95% confidence interval based on the Wilson's score method (Wilson 1927). Bacterial quantification data was entered into Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) for descriptive statistical analyses. The relationship between sow parity and qPCR status (positive or negative) was evaluated using a using a two-tailed chi-square linear-by-linear association test, with stratification by herd. Due to small counts, the p-value was computed through Monte-Carlo resampling using 100,000 iterations. The statistical analyses and graphical representations were carried out using the statistical software R (R Foundation for Statistical Computing, Vienna, Austria) with the coin package (Hothorn, et al, 2008).

3.4 Results

3.4.1 Study populations

Three farrow-to-wean herds, defined as A, B and C and their nurseries located in southern Minnesota and southeast South Dakota were included in this study (Table 1). These multisite herds were part of one production system with the same genetic, nutrition and biosecurity protocols. Sows from herd C were housed in two breeding herds geographically separated (<1.2 miles). Weaning was done in off-site wean-to-finish facilities with all-in all-out flows by barn. In herd C, weaned piglets from both farms

were commingled during placement at nursery sites. Herd A and B were experiencing approximately 5% nursery mortality associated with *M. hyorhinis*. At the time of the study, herd C had no clinical cases of *M. hyorhinis* infection.

3.4.2 Prevalence of *M. hyorhinis* infection

3.4.2.1 Breeding herd

In each herd a total of 60 sows, 240 pre-weaning pigs and 240 post-weaning pigs were sampled and tested by qPCR for the presence of *M. hyorhinis* in the nasal cavity. In herd A and B, the prevalence of *M. hyorhinis* infection in sows was 8.8% (5/60, 95% CI: 3.6-18.1%) and 3.3% (2/60, 95% CI 1.7-13.7%), respectively. In contrast, all sows from herd C were negative by qPCR (0/60, 0%, 95% CI: 0-6%) (Fig.1). The median bacterial load in the nasal cavity for sows in herd A and B was 4.5 and 3.9 log10 geq/swab, respectively (Fig. 2). The linear-by-linear association test revealed a relationship between the sow parity and presence of *M. hyorhinis* in the nasal cavity. Younger sows were more likely to test positive by qPCR in the nasal cavity (chi-squared= 5.7789, p-value= 0.018) (Table 2).

Prevalence of *M. hyorhinis* infection in pre-weaning piglets was low in all three herds (Fig. 1). In herd A, 10% (6/60, 95% CI: 4.7-20.9%) of piglets were positive shortly after birth. Similar findings were observed for piglets at 7 (5/60, 8.3%, 95% CI: 3.6-

18.1%), and 21 days (4/60, 6.7%, 95% CI: 2.6-16.1%). All piglets of 14 days were negative by qPCR (0/60, 95% CI: 0-6%). In herd B, prevalence shortly after birth and at 14 days was 5% (3/60, 95% CI: 1.7-13.7) and 6.7% (4/60, 95% CI: 2.6-16.1%) at 21 days. All piglets of 7 days were negative by qPCR (0/60, 95% CI: 0-6%). In herd C, all piglets of 1, 7, and 14 days were negative and only 1.7% (1/60, 95% CI: 0.3-8.9) of piglets of 21 days were positive by qPCR. Median log10 geq/swab values ranged between 3.7 and 6.6 (Fig. 2).

3.4.2.2 Nurseries

In herd A, the prevalence of infection in pigs was 53.5% (16/30, 95% CI: 36.1-69.8%) at 28 days, 40% (12/30, 95% CI: 24.6-57.7%) at 35 days, 96.7% (29/30, 95% CI: 83.3-99.4%) at 42 days and 100% (30/30, 95% CI: 88.6-100%) at 49 through 77 days of age. In herd B, prevalence of *M. hyorhinis* infection was close to or at 100% in all sampled age groups (Fig. 1). In contrast in herd C, only two pigs were positive prior to 77 days of age, after which the prevalence reached 100% (30/30, 95% CI: 88.6-100%). Median log10 geq/swab values ranged between 6.6 and 9.35 (Fig. 2).

3.4.3 Oral fluids

M. hyorhinis was detected by qPCR in all oral fluid samples in herds A and B with the exception of samples collected from pigs at 35 days in Herd A and 28 days in herd B. In both cases most pigs did not seem to interact with the rope, thus very little volume was

obtained (<2 ml). In herd C, 1/8 oral fluids sample was positive at 77 days. The bacterial load of these samples ranged from 3.4 - 6.7 log10 geq/ mL.

3.4.4 Clinical and pathological evaluations

In herds A and B fibrinous polyserositis was observed in 9/10 and 4/10 diseased pigs, respectively by histological examination. *M. hyorhinis* was detected by qPCR in the pericardium of 8/10 diseased pigs in herd A and 3/10 in herd B (Table 3). In herds A and B, *M. hyorhinis* was detected by qPCR in the pericardium of 2/10 and 1/10 healthy pigs with no lesions, respectively. Isolation of *M. hyorhinis* from the pericardium was achieved only in herds A (2/10) and B (3/10). In herd A, histological examination revealed fibrinous and proliferative arthritis in 7/0 pigs and 4/10 of those with lesions were positive for *M. hyorhinis* by qPCR. Joint lesions were not seen in pigs from herd B, but 1/10 diseased pigs tested positive in the joint for *M. hyorhinis* by qPCR (Table 3). Lesions suggestive of fibrinous polyserositis and arthritis were not observed in any of the healthy pigs from all three herds or in the diseased pigs from herd C. *M. hyorhinis* was detected in bronchial swabs in 7/10 diseased and 1/10 healthy pigs from herd A, and in 6/10 diseased and 10/10 healthy pigs from herd B. *M. hyorhinis* was not detected by PCR in any of the necropsied pigs from herd C (Table 3).

3.5 Discussion

M. hyorhinis is considered to be ubiquitous in swine (Friis and Feenstra, 1994; Gois et al., 1968). However, there is very limited knowledge regarding infection frequency and age distribution of this bacterium in modern swine production. The objective of this study was to estimate the prevalence of *M. hyorhinis* infection in pigs from different age groups in three commercial swine populations. The results of this study showed that the prevalence is very low in sows and pre-weaning piglets and very high by the end of the nursery period. In herds A and B, quantitative PCR testing on nasal swabs revealed a low proportion of positive sows. Similar findings were observed in a previous study where *M. hyorhinis* was isolated from nasal secretions of 2/27 and from frontal sinus secretions of 1/27 euthanized sows of 1 to 3 years of age (Ross and Spear, 1973). Interestingly, there was a significant relationship observed between parity and qPCR status of the sow indicating that young sows are more likely to be positive. These results may indicate a possible role of the sow as a source of infection for suckling piglets

In herds A and B, *M. hyorhinis* was detected in a few piglets shortly after birth. However, *M. hyorhinis* does not seem to spread quickly at this age and overall pre-weaning prevalence was relatively low (<10%). In herd C only 1/240 pre-weaning piglet was positive by qPCR. There are no published studies reporting *M. hyorhinis* prevalence estimates for suckling piglets. Such low prevalence could be due to low exposure to the

bacterium in the farrowing room environment and to protective effects of colostral immunity. Future studies should explore the role of colostral immunity and piglet colonization. The low prevalence of *M. hyorhinis* infection estimated in this study is in contrast with the high pre-weaning prevalence (>80%) of other commensal swine bacteria in the nasal cavity, such as *Haemophilus parasuis* and *Streptococcus suis* (Cerdà-Cuéllar et al., 2010; Amass et al., 1995; Amass et al., 1996). However, similar findings to those presented here, have been reported for *M. hyopneumoniae* and *M. hyosynoviae* (Ross and Spear, 1973; Fano et al., 2007)

In herd A, the prevalence of infection was around 50% during the first two weeks post-weaning. In older groups (ages 42 to 77 days of age), prevalence was 90-100%. An earlier increase was observed in herd B, where most pigs were colonized shortly after placement in the nurseries. These results are in agreement with the limited information available in the literature. In one study, *M. hyorhinis* was isolated from the nasal cavity of 56/61 grower pigs (30kgs) in Czechoslovakia (Gois et al., 1968). The increase of *M. hyorhinis* prevalence shortly after weaning may be due to the decay of colostral immunity, commingling of pigs at weaning, co-infections such as with porcine reproductive and respiratory syndrome virus (PRRSv) (Kobayashi et al., 1996) and exposure of pigs with *M. hyorhinis* contaminated facilities. The high prevalence of *M. hyorhinis* in herds A and B was in contrast with the prevalence observed in herd C, where *M. hyorhinis* was not detected until 77 days of age. This latter observation shows that,

although *M. hyorhinis* is considered a commensal organism, not all pigs in a population carry the bacterium in the nasal cavity at a particular time. Quantification of the bacterial load, although its significance is still unclear, may provide useful information regarding trends of infection.

Three different patterns were seen in this study possibly explained by several factors. In herd C, lower prevalence estimates were observed at each age group and no *M. hyorhinis* disease was identified, compared to herds A and B. One of the differences of this herd compared to herds A and B is that it is a parity-segregated system, where parity 1 sows are housed in geographically separated buildings from those housing parity 2 and older sows. Such separation might have affected the dynamics of infection in herd C. Although all three herds were under the same management, the differences in timing of strategic medications for each herd could have impacted the temporal prevalence pattern. Specifically, the use of tulathromycin in herd A could have slowed down the spread of *M. hyorhinis* in the nursery, compared to herd B. The impact of such antibiotic treatment on *M. hyorhinis* infection dynamics should be addressed in the future. Another factor that could help explain some of the differences between herds is the presence of coinfections with other pathogens. For example, the increased nursery mortality and proportion of pigs with *M. hyorhinis* disease observed in necropsies in herd A compared to herd B could be due to coinfection with PRRSv. Other studies have found an increase in isolation of *M. hyorhinis* in lungs from pigs infected with PRRSv (Shimizu et al., 1994; Kawashima et

al., 1996; Kobayashi et al., 1996). Furthermore, differences in the disease presentation and infection patterns between herds may be due to differences in the virulence of *M. hyorhinis* strains present in each herd. Differences in the virulence of *M. hyorhinis* strains have been previously described (Gois et al., 1971; Gois and Kuska, 1974; Shulman et al., 1970)**.** Finally, differences in gilt source and flow could have played a role in the colonization patterns observed between herds. In previous studies, two genetic lines displaying different immune responses have shown different susceptibility to *M. hyorhinis* disease after experimental inoculation (Magnusson et al., 1998). Future studies addressing potential risk factors for *M. hyorhinis* disease, as well as variability in temporal prevalence patterns are needed.

The detection of *M. hyorhinis* in oral fluids followed similar patterns as those observed using nasal swabs. The negative qPCR results obtained from oral fluids at 28 and 35 days for herds A and B, respectively, can be explained by the lack of engagement of most of the pigs with the collection rope at those sampling times, which resulted in a low volume of oral fluids, probably representing only a few pigs. Although more validation is required, oral fluids appear to be a good tool for *M. hyorhinis* surveillance.

Determining the role of *M. hyorhinis* in systemic disease from clinical cases can be challenging since it is an organism that colonizes the upper respiratory tract of both

healthy and diseased pigs (Friis et al., 1971). In this study, there was the unique opportunity of necropsying both affected and unaffected pigs from the same population. While *M. hyorhinis* was detected in the nasal cavities of both healthy and diseased pigs, detection in systemic sites was mainly observed in diseased pigs and with a strong association with the presence of fibrinous serositis. Therefore, these results support the role of *M. hyorhinis* as a cause of systemic disease, as previously demonstrated in several experimental inoculation studies (Roberts et al., 1963; Duncan and Ross, 1969; Barthel et al., 1972; Friis and Feenstra, 1994).

Limitations of this study arise mainly from the intrinsic characteristics of a cross-sectional study. In this type of study different sets of pig populations are sampled in each age category, in contrast to the frequent sampling of piglets over time in a longitudinal study; however, this is the first description of the prevalence of *M. hyorhinis* across multiple age groups of pigs from commercial swine populations. Results from this study suggest that a few sows are carriers of the bacterium, which is in turn transmitted to a few piglets. These infected piglets would be the source for transmission to the rest of the pigs during the nursery period. However, this can only be proved with a longitudinal study that follows the same pigs over time. In addition, this study suggests that pigs get infected a couple of weeks before they become sick. This would imply that pigs that get colonized in the nasal cavity do not necessarily develop a protective immune response. Therefore, there is a need for a longitudinal study that can test these hypotheses and help elucidate

the role of infected sows, infected pigs and the immune response in the dynamics of *M. hyorhinis* infection in affected herds.

Table 3.1 Breeding herd and nursery site characteristics.

	Herd A	Herd B	Herd C
Breeding herds			
Herd size (n)	6000	6000	6000
Herd Location (state)	MN	SD	MN
Production type	Farrow-to-wean	Farrow-to-wean	Farrow-to-wean
Gilt source/management	A	B	B
PRRS Status	Positive	Negative	Negative
M. hyopneumoniae status	Positive	Positive	Positive
Antibiotic treatments:			
Piglets	Tulathromycin (day 4 and weaning)	Penicillin (day 4)	Penicillin (day 4)
Sows	Spot treatment tylosyn, lincomycin and penicillin	Spot treatment tylosyn, lincomycin and penicillin	Spot treatment tylosyn, lincomycin and penicillin
Vaccination protocol:			
Piglets	PCV2, *H. parasuis, A. suis, Salmonella, F-18 E. coli, Mycoplasma, H. parasuis*	PCV2, *H. parasuis, A. suis, Salmonella, F-18 E. coli, Mycoplasma, H. parasuis*	PCV2, *H. parasuis, A. suis, Salmonella, F-18 E. coli, Mycoplasma, H. parasuis*
Sows	PRRS, Rotavirus, SIV, E. coli-Clostridium, *E. Rhusiopathiae*	PRRS, Rotavirus, SIV, E. coli-Clostridium, *E. Rhusiopathiae*	Rotavirus, SIV, E. coli-Clostridium, *E. Rhusiopathiae*
Weaning age	19-21	18-21	19-21
Pre-weaning mortality rate (%)	15.3	13	12.5
Nurseries			
Sites	3	3	3
Location	MN, IA	MN, IA	MN, IA
Production type	Wean-to-finish	Wean-to-finish	Wean-to-finish
Pig Flow	AIAO	AIAO	AIAO
Capacity per site	8800	6600	6600
Stocking density (m^2/animal)	0.3	0.3	0.3
Number of pigs per pen	75	75	75
Strategic antibiotic treatments	Tulathromycin (day 35 and 42) tiamulin/CTC	tiamulin/CTC (feed) tiamulin/OTC	tiamulin/CTC (feed) tiamulin/OTC

	(feed) tiamulin/OTC (water)	(water)	(water)
Vaccination protocol	E.coli, PCV2, *M. hyopneumoniae, L. intracellularis*	E.coli, PCV2, *M. hyopneumoniae, L. intracellularis*	E.coli, PCV2, *M. hyopneumoniae, L. intracellularis*
Mortality rate (%)	4.4	3.7	2.8

Figure 3.1 Prevalence of *M. hyorhinis* nasal infection and 95% confidence interval per age category.

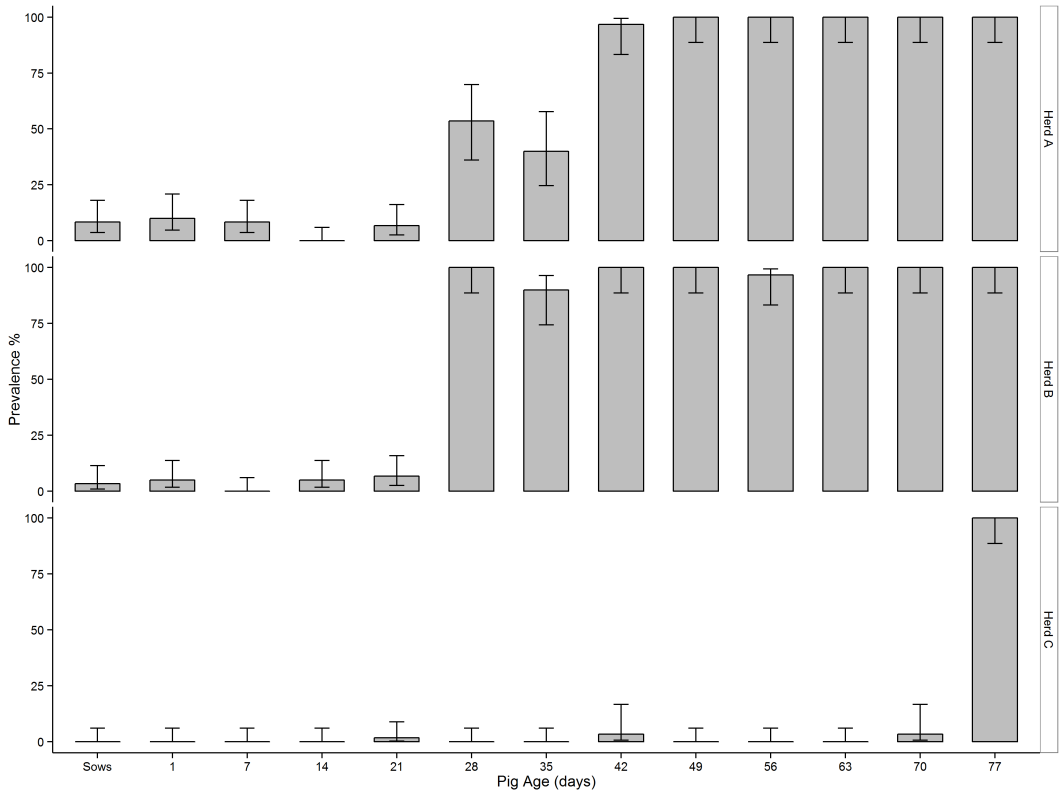


Table 3.2 Prevalence of sow nasal infection by parity.

	Herd A				Herd B				Herd C			
Parity	**Total (n)**	**%**	**qPCR + (n)**	**% pos**	**Total** (n)	**%**	**qPCR + (n)**	**% pos**	**Total (n)**	**%**	**qPCR + (n)**	**% pos**
1	11	18	2	18	18	30	2	11	8	13	0	0
2	9	15	2	22	12	20	0	0	15	25	0	0
3	15	25	0	0	5	8	0	0	10	17	0	0
4	11	18	1	9	8	13	0	0	10	17	0	0
5	7	12	0	0	5	8	0	0	6	10	0	0
6	4	7	0	0	10	17	0	0	7	12	0	0
7	3	5	0	0	2	3	0	0	3	5	0	0
8	0	0	-	-	0	0	-	-	1	2	0	0
Total	60	100	5	-	60	100	2	-	60	100	0	-

Figure 3.2 *M. hyorhinis* bacterial load of pigs of different age categories. The upper and lower hinges correspond to the 25th and 75th percentiles, respectively. The hinge within the box represents the median. Whiskers extend from each hinge to the highest and lowest value that are within 1.5 * the inter-quartile range. Data not covered by whiskers are outliers and are represented by points (McGill et al., 1978).

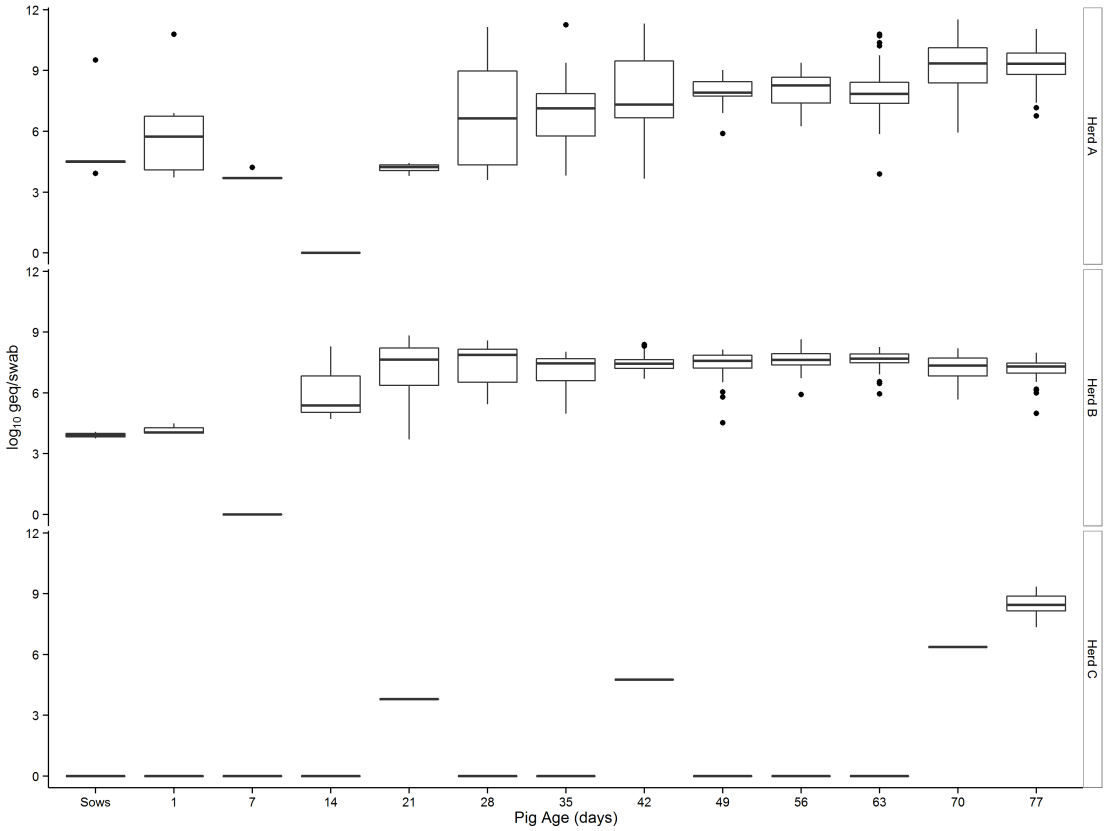


Table 3.3 Diagnostic evaluation of diseased pigs**.**

Pathogen	Diagnostic test	Type of sample	Herd A	Herd B	Herd C
M. hyorhinis	qPCR	Pericardium	8/10	3/10	0/10
		Joint	4/10	1/10	0/10
		Bronchus	7/10	6/10	NT
	Mycoplasma culture	Pericardium	2/10	3/10	0/10
		Joint	0/10	0/10	0/10
H. parasuis	PCR	Pericardium	3/10	6/10	2/10
		Joint	1/10	0/10	0/10
	Aerobic culture	Pericardium	0/10	0/10	010
		Lung	0/10	1/10	0/10
S. suis	Aerobic culture	Lung	2/10	0/10	0/10
		Pericardium	2/10	0/10	0/10
		Liver	2/10	0/10	0/10
P. multocida	Aerobic culture	Lung	0/10	0/10	5/10
Salmonella sp	Aerobic culture	Lung	1/10	0/10	1/10
		Pericardium	0/10	0/10	2/10
		Liver	1/10	0/10	0/10
M. hyopneumoniae	PCR	Bronchus	0/10	0/10	0/10
M. hyosynoviae	PCR	Joint fluid or tissue	0/10*	0/10	0/10
NAEU-PRRSv	qPCR	Tissue homogenate (1 pool= 5 pigs)	2/2	0/10*	0/2
Influenza A	PCR	Lung (1 pool= 5 pigs)	0/2	0/10*	0/2
PCV2	PCR	Tissue homogenate (1 pool= 5 pigs)	0/2	2/10*	0/2

NT- Not tested

* Tested individual animals

CHAPTER IV
INFECTION DYAMICS OF *MYCOPLASMA HYORHINIS* IN TWO THREE-SITE SWINE PRODUCTION SYSTEMS

Clavijo MJ, Davies P, Morrison R., Bruner L, Olson S, Rosey E, Rovira A. Infection dynamics of *Mycoplasma hyorhinis* in two three-site swine production systems. [in prep, to be submitted to Journal of Veterinary Research]

4.1 Summary

Appropriate control of *Mycoplasma hyorhinis* associated disease is currently hindered by a lack of knowledge on epidemiological and ecological factors related to this organism. A prospective longitudinal investigation on sows and piglets was carried out to investigate the dynamics of *M. hyorhinis* infection in two swine production systems. In each system, A and B, a longitudinal sampling of pigs at different ages was performed. Fifty one young sows (p1 and p2) and 56 old sows (p3 and older) were randomly selected and tested for *M. hyorhinis* by nasal swab qPCR and *M. hyorhinis* antibodies in serum by ELISA. One piglet per litter was randomly selected from each sow. A nasal swab and a serum sample were collected from each pig at birth, weaning and 10 days post-weaning. Two final samplings were performed in the nursery and finishing stage during the peak of polyserositis/ arthritis/ pneumonia. A total of twelve pigs were euthanized and necropsied (10 clinically diseased and 2 clinically healthy) during these two sampling points. Oral fluids were also collected at each post-weaning sampling point. The prevalence of *M. hyorhinis* colonization in sows was low (<5%), confirming results from our previous study. No correlation was found between sow parity or sow serum titer and piglet nasal colonization at birth or at weaning. In contrast to the low prevalence observed in pre-weaning piglets, most pigs became colonized during the first four weeks in the nursery and remained positive throughout the nursery and finishing stage. This is the first study to show that pigs can remain positive for *M. hyorhinis* for most of the post-weaning phase. Testing oral fluids to detect *M. hyorhinis* appears to be useful for surveillance, but

additional validation is required. ELISA results showed decay in maternal antibodies at around 3 weeks of age and a subsequent increase after natural infection. The role of *M. hyorhinis* in polyserositis and arthritis was demonstrated in these two herds. The knowledge of the dynamics of infection within the herd will allow implementation of better control strategies in affected herds.

4.2 Introduction

Mycoplasma hyorhinis (*M. hyorhinis*) belongs to the mollicutes class, which comprises more than 100 mycoplasma species that infect humans and animals (Razin et al., 1998). This bacterium is considered a commonly occurring microorganism in swine populations. The natural habitat for *M. hyorhinis* is the mucous membrane of the upper respiratory tract of pigs and tonsils. However, it is frequently isolated from lungs of healthy pigs (Friss and Feenstra, 1994; Kobisch and Friis, 1996). This wall-less bacterium is one of the causative agents of polyserositis and arthritis in post-weaning pigs and it has also been linked to eustachitis (Morita et al., 1999) and pneumonia (Gois et al., 1968; Gois et al., 1971; Poland et al., 1971; Kinne et al., 1991). The detection of *M. hyorhinis* is mainly achieved through PCR or bacterial isolation. Although several serological assays have been developed to detect antibodies against this pathogen, these are not commercially available (Roberts et al., 1963; Gois M, 1967; Poland et al., 1971; Gois et al., 1972; Gois et al., 1972).

Knowledge related to the epidemiology of *M. hyorhinis* in commercial swine populations is sparse. *M. hyorhinis* can be transmitted through direct contact between infected and naïve pigs. In chapter three, the prevalence of infection in pigs of different ages from three commercial swine herds was estimated. In that study, the prevalence of infection in sows and pre-weaning piglets was low, with less than 10% of sows and piglets infected. This contrasted with the noticeable increase in prevalence after weaning,

which reached 100% of the pigs by the end of the nursery period. Therefore, the study supported the hypothesis that a low proportion of young positive sows wean positive piglets, which become the source of infection for other pigs after weaning. However, that study was an observational cross-sectional study. The limitations of a cross-sectional study include measuring exposure and outcome (infection) at the same time point. In addition, each sampled age group is a distinct population, thus, differences observed might be due to unknown factors within each group and not to the age differences. These limitations emphasized the need for a longitudinal study in order to investigate the previously stated hypothesis by following the same pigs over time.

The general objective of this chapter was to investigate the infection dynamics of *M. hyorhinis* in affected herds through a longitudinal study. The specific objectives of this study were (1) to determine the prevalence and incidence of *M. hyorhinis* in naturally infected pigs over time and (2) to evaluate the relationship between sow colonization and serological status with piglet infection.

4.3 Materials and methods

4.3.1 Study design

All protocols of pig care and management were approved, prior to the beginning of the study, by the University of Minnesota Institutional Animal Care and Use Committee (IACUC protocol number 1006A84138). The study was a prospective cohort study in

which a nasal swab and a blood sample from young (p1 and p2) and old (p3 and above) sows were collected shortly after farrowing. From each sow a piglet was selected and tagged shortly after birth. A nasal swab and a blood sample were collected from the selected pigs multiple times during their life. In each system, *M. hyorhinis* associated disease was studied by means of post-mortem investigation of a set of pigs during the peak of mortality in the nursery and finishing stages.

4.3.2 Selection of systems

System enrollment began in May of 2012. System selection criteria were: (1) three-site production system, (2) breeding herd with at least 1500 sows, (3) recent cases of *M. hyorhinis* associated disease diagnosed by PCR or culture, (4) no vaccination against *M. hyorhinis,* (5) location within driving distance to the University of Minnesota Saint Paul Campus, and (6) willingness to collaborate in a long-term project. Seven different swine systems that fulfilled conditions 1, 2 and 3 were investigated as candidates for the study. One system was ineligible due to the use of a *M. hyorhinis* autogenous vaccine. Two other systems were too distant from the University of Minnesota. Two other systems were eligible but either the veterinarian or the owner decided not to participate. Two other systems fulfilled all the selection criteria and were enrolled in the study. These systems, A and B, belonged to two different commercial pork production companies located in swine dense regions in southern Minnesota and followed industry standard

biosecurity practices. Prior to the sampling, the presence of *M. hyorhinis* was confirmed in both systems by performing necropsies on diseased post-weaning pigs. The study began in September 2012 and ended in December 2012 for system A, and began in October 2012 and ended February 2013 for system B.

4.3.3 Production systems

4.3.3.1 System A

The farrow-to-wean breeding herd had 4,700 sows housed in buildings connected through hallways with 16 farrowing rooms managed all-in-all-out (AIAO) by room. Gestating sows were housed in individual crates and gilts were housed in pens. The replacement gilts were obtained through internal multiplication and were moved every month into gilt development units (GDUs) and from the GDUs into the breeding barn every week. The piglets were weaned at 18-21 days and kept on site to be shipped out twice a week. At the time of the study the farm was weaning porcine reproductive and respiratory syndrome virus (PRRSV) negative pigs. Pigs were transported to off-site double stocked wean-to-finish facilities composed of one building and two rooms with 40 pens each. These sites commingled pigs originated from multiple breeding herds. However, all study pigs were housed in the same room, separated from pigs from other sources by a solid wall and a hallway. After seven weeks post placement in the nursery the study pigs were transported to an AIAO multisource finishing facility. All pigs were housed in two buildings with 2

rooms per building separated by a solid wall. Post-weaning facilities commingled pigs from 5 different sow herds (Table 1). Study pigs were housed together with their cohorts and were not commingled with other pigs originating from different sow herds.

4.3.3.2 System B

The breeding herd from system B housed 6,000 sows in two large interconnected buildings with 21 farrowing rooms managed AIAO by room. All sows and gilts were housed in crates during all production stages. Piglets were weaned at 17-21 days of age two to three times per week and transported to off-site nursery sites. This system was weaning PRRSV positive pigs. The single source nursery facilities were comprised of two barns housing 6,000 pigs in 6 or 12 rooms with a capacity of 1000 or 500 pigs, respectively. There were between 20 and 40 pens in each room. The pigs were managed AIAO by barn and remained at these facilities for 7 weeks, after which they were transported to single source AIAO finishing sites comprised of two 1,000-head barns or one 300-head barn (Table 1).

4.3.4 Sampling protocol

4.3.4.1 *M. hyorhinis* nasal infection

Breeding herds

In chapter three a relationship was observed between the parity of the sow and the quantitative PCR (qPCR) status. Younger sows were more likely to be positive by qPCR in the nasal cavity. Therefore, to identify possible differences of *M. hyorhinis* infection patterns in pigs born from sows of different ages, the sows were separated into young (p1 and p2) and old (p3 and above). A total of 51 young sows and 56 old sows were selected shortly after farrowing (Figure 1). Sample size was estimated based on results from chapter three, where there was a higher proportion of *M. hyorhinis* qPCR positive young sows (20%) compared to old sows (2.5%). Sample size estimation to compare proportions assumed a relative risk of 8, a confidence level of 95% and a power of 80% (*Sample Size Calculator for a Cohort Study,* AusVet - Animal Health Service, Australia). A bilateral nasal swab and a blood sample were collected from each sow. One piglet per litter was selected by simple randomization from each previously selected sow, for a total of 107 piglets. Piglets were ear tagged and a bilateral nasal swab and a blood sample were obtained, within 24 hours after birth. A nasal swab sample was collected again from each pig right before weaning. For system B, a blood sample was also obtained at the same time (Figure 1). Piglets selected in this study were not cross-fostered. Antibiotic treatments and parity of sows and piglet gender were recorded. None of the monitored piglets received preventative antibiotic treatments in the farrowing room.

As a side objective, the presence of *M. hyorhinis* in the environment where suckling piglets were kept was investigated as a potential source of indirect transmission of *M. hyorhinis*. Recently the use of cloth pads (Swiffer® cloths) has been used to detect

PRRSV in the environment (Kenney et al 2011). A total of twelve cloth pads (Swiffer® cloths), previously soaked in D-PBS, were used to sample the floors, walls, bars and feeders of farrowing crates with pigs (n=10) and without pigs (n=2) in each herd at weaning. Sampled crates housing study pigs were chosen by simple randomization.

Growing pig sites

A nasal swab and blood sample were collected from each tagged pig 10 days after placement into the nursery. Each pig was sampled again in two additional occasions, one during the nursery period and one more during the finishing period. These latter sampling times were scheduled to capture the peak of polyserositis/arthritis/pneumonia in the nursery and finishing stage based on clinical signs reported by the herd veterinarian and historic mortality records. Twelve of these pigs from each herd were euthanized and necropsied for post-mortem investigation of the causes of disease (Figure 1).

4.3.4.2 Oral Fluids

The usefulness of oral fluids for *M. hyorhinis* detection was evaluated by collecting a set of oral fluid samples at each post-weaning sampling time (described in previous section).

In each system, between 7 and 12 oral fluid samples were collected from pens containing the pigs in the study. Non-bleached cotton ropes were suspended at the pig's shoulder level in each pen. All tagged pigs and their pen mates had access to the rope for approximately 45 minutes, after which the rope was introduced into a plastic bag and the oral fluid was removed from the rope by squeezing the wet portion. The obtained liquid was then placed into a plastic sterile tube and stored at -80 °C until testing for *M. hyorhinis* by PCR (Prickett et al., 2008).

4.3.4.3 Clinical signs and postmortem observations

In each system, post-mortem investigations were carried out to determine the status of *M. hyorhinis* associated disease in the study pigs. The attending veterinarian considered the current clinical picture, as well as previous mortality rates to estimate the ideal time for the investigations. A total of ten tagged pigs with clinical signs indicative of polyserositis (reluctance to move, cough, lameness, dyspnea and fever) and two clinically healthy tagged pigs were humanly euthanized, and a complete necropsy was performed. Pleural, pericardium and synovial surfaces were swabbed and were examined for *M. hyorhinis* by qPCR, for *Haemophilus parasuis* by PCR and for aerobic bacteria by bacterial culture. In addition, liver and lung tissue samples were used for aerobic bacterial culture. A set of aseptically collected samples from lung, heart, spleen, kidney, liver and inguinal lymph node were obtained from each pig and fixed in 10% formalin for histological inspection.

PRRSV and porcine circovirus type 2 (PCV2) PCRs were performed on a tissue pool containing lung, spleen and inguinal lymph node. Bronchial swabs were tested for *Mycoplasma hyopneumoniae* and *M. hyorhinis* by PCR and a lung sample was utilized for detection of *Influenza A* virus (IAV) by PCR. In addition, a subset of the monitored pigs (9) from system A was sampled at slaughter. From each pig a tonsil and a joint swab were collected. Due to the logistics at the slaughter plant, nasal swabs could not be collected.

4.3.5 Diagnostic testing

4.3.5.1 Bacteriological culture

All swabs collected during the necropsies and a subset of 36 nasal swabs from sows (n=10), piglets (n=10) at birth and at finishing (n=16) from system B, were placed into tubes containing 3 mL of Hayflick's liquid media and agitated to release the sample into the media (Lefebvre et al., 1987), followed by placing 150 µl of the mixture onto Hayflick's agar media. Samples in liquid media were incubated aerobically and samples in agar media were incubated with 5% CO_2. After 2-14 days at 37°C, isolates were stored at -80 °C following confirmation of growth in agar media.

4.3.5.2 Sample processing and quantitative PCR

Oral fluids and nasal, serosal and bronchial swabs collected from sows and pigs were stored at -20 °C until testing was performed. Swabs were immersed in 0.5 mL of D-PBS

(Sigma-Aldrich, St Louis, MO). The suspension was vortexed and centrifuged and DNA was extracted from the pellet using the DNeasy Blood & Tissue kit (Qiagen, Germantown, MD). All samples were tested by a TaqMan® qPCR targeting the 16S rRNA gene of *M. hyorhinis* (Chapter two, Clavijo et al., submitted 2014). The qPCR reaction was prepared in a volume of 25 µl consisting of 5 µl of the template DNA, 12.5 µl of Master Mix solution, 1 µl of ROX dye, 0.125 µl of each 40 µM reverse and forward (primers, 1.25 µl of 5 µM TaqMan probe® (5'-FAM-CGG ATA TAG TTA TTT ATG CGC A-TAMRA-3') and 5.0 µl of DNAse free H_20. Reactions were carried out in the ABI 7500 fast real-time PCR system (Life Technologies, Grand Island, NY) at 95°C for 3 min, 35 cycles of 95°C for 15 sec and 54°C for 50 sec. Individual samples were run in duplicate and each run contained a negative and positive extraction control as well as a PCR negative control (H_2O).

4.3.5.3 Serology

Blood samples were collected by jugular vein puncture and the serum was separated by centrifugation and stored at -20^o C until testing. An ELISA protocol to detect *M. hyorhinis* was developed and optimized as follows. High protein-binding capacity polystyrene 96 well ELISA plates (MaxiSorp^{TM}, NUNC, Roskilde, Denmark) were coated overnight with whole-cell antigen from a US clinical isolate of *M. hyorhinis* diluted 1:400 in carbonate/bicarbonate buffer (Sigma Aldrich, St. Louis, MO, USA) at 4^o C and blocked with 5% fish gelatin (Sigma Aldrich, St. Louis, MO) in phosphate

buffered saline (Gibco, Carlsbad, CA, USA) containing 0.05% Tween 20 (Sigma Aldrich, St. Louis, MO) at 37^{o} C for 1 hour. Serum samples were serially diluted 1:100 to 1:3200 (two-fold dilutions) in blocking buffer using a robotic liquid handler (Biomek 3000, Beckman Coulter, Brea, CA, USA) and added to the ELISA plates along with positive and negative control sera diluted 1:100 in blocking buffer. Sealed plates were incubated at 37^{o} C for 1 hour followed by three washes with phosphate buffered saline containing 0.05% Tween 20. An HRP-conjugated goat anti-swine IgG(H+L) antibody (Kierkegaard and Perry Laboratories, Gaithersburg, MA, USA) was diluted 1:1000 in blocking buffer and added to each well followed by a 1 hour incubation at 37^{o} C. Plates were then washed as described and developed with TMB peroxidase solution (Kierkegaard and Perry Laboratories). Optical density (OD) was measured at 405 and 490 nm (Spectramax Plus, Molecular Devices, Sunnyvale, CA, USA). For each sample and each dilution, a corrected OD value was calculated by subtracting the OD obtained at 490 nm from the OD obtained at 405 nm. Samples were considered positive when higher than the corrected OD for the negative control plus 2 standard deviations. The titer of each sample was calculated as the maximum dilution yielding a positive result.

4.3.5.4 Statistical analysis

Incidence rate (new cases per pig days at risk) is defined as the number of new cases (infected) in a population per unit of animal-time during a given time period (Dohoo et

al., 2003). Incidence rates and confidence intervals were calculated for each time period between two samplings, for a total of four time periods per system. The equation for incidence rate was:

$$IR\ (time\ 1 - time\ 2) = \frac{new\ cases}{\{pig{-}days\ at\ risk\}} * 100$$

Apparent prevalence was estimated as the number of positive pigs divided by the total number of sampled pigs in the nasal cavity. The 95% confidence intervals for each prevalence estimate and for the proportions of positive oral fluid samples were calculated using the Wilson's score method (Wilson 1927). Bacterial load, expressed as geq/swab or geq/mL of oral fluid, and serology data, expressed as serum titer were entered into Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) and exported to R for statistical analysis on log transformed data. Graphical analysis was carried out using dot plots and box plots. The proportion of positive sows between groups (young and old) and the proportion of positive piglets from young and old sows were compared using a Fisher's exact test.

4.4 Results

4.4.1 *M. hyorhinis* nasal infection

4.4.1.1 Breeding herd

A total of 107 and 111 piglets and sows were sampled in system A and B, respectively. Pre-weaning mortality was 9% in system A and 17% in system B, resulting in 97 and 92 pigs remaining at weaning, respectively. A low proportion of *M. hyorhinis* PCR-positive piglets in the nasal cavity was observed prior to weaning. In system A, only 1/107 piglet (0.93%, 95% CI: 0.2-5.1%) was positive prior to weaning and in system B 3/111 piglets were positive at birth (2.70%, 95% CI: 1-8%), and 3/92 piglets were positive prior to weaning (3.26%, 95% CI: 1-9%) (Figure 2). Piglets positive at birth either tested negative at weaning or died before weaning. In both systems, a higher bacterial load in the nasal cavity was observed in pigs 10 days after placement in the nurseries compared to pigs prior to weaning (Figure 3). In system A, the bacterial load of the piglet that tested positive at birth was 3.8 $\log_{10}$ geq/swab. In system B, the range of the bacterial load in positive piglets at birth was 3.6-5.0 $\log_{10}$ geq/swab, and 3.7-7.6 $\log_{10}$ geq/swab in positive piglets at weaning.

Similarly, the prevalence of *M. hyorhinis* infection in sows was low. *M. hyorhinis* was detected in 5/107 sows (4.67%, 95% CI: 2-11%) from system A and 2/110 sows (1.8%, 95% CI: 0.5-6.4%) from system B (Table 2). The median bacterial load for sows in system A was 3.8 geq/swab, while in system B, the bacterial load for the two positive sows was 4.6 and 4.7 $\log_{10}$ geq/swab. There was no statistical difference between the proportion of positive young sows and the proportion of positive old sows. However, in system B, positive results were only found in parity 1 sows. In system A, the parities of

the positive sows were 2 (n=2), 3(n=1) and 6 (n=2). All positive piglets at birth originated from negative sows while 1/3 positive piglets at weaning originated from positive sows at farrowing in system B. There was no significant difference between the proportions of positive piglets from young or old sows.

4.4.1.2 Grower pigs

In contrast to the low prevalence observed in pre-weaning piglets, most pigs became colonized during the fist four weeks in the nursery and remained positive throughout the nursery and finishing stage (Figure 2). In system A, 20/93 pigs (22%, 95% CI: 14-31%) and in system B 82/82 pigs (100%, 95% CI: 96-100%) were positive by qPCR at 10 days after placement in the nursery (29 and 27 days of age, respectively).

In each system, the attending veterinarians determined the final two sampling times, based on clinical signs and mortality data from previous cohorts. For system A 85/90 sampled pigs (94% (95% CI: 88%-98%) tested positive by qPCR at 47 days of age and 84/84 (100%, 95% CI: 96-100%) were positive at 94 days of age. In system B, 80/80 (100%, 95% CI: 94-100%) were positive at 64 days of age and 48/50 (96%, 95% CI: 87-99%) of pigs were positive at 140 days of age. The decrease in number of sampled pigs from 80 to 50 at 140 days was due to the loss of ear tags. In system A, the range of bacterial load was 3.7-7.8, 3.6-7.8 and 4.3-8.3 $\log_{10}$ geq/swab at 29, 47 and 94 days of age, respectively. Similar results were observed in system B (Figure 3).

The incidence rate was estimated for the different phases between samplings (Figure 2). In system A, from 19 to 29 days of age the incidence rate was 2 new cases per 100 pig-days (95% CI: 1-3 new cases per 100 pig-days). The incidence increased to 5 new cases per 100 pig-days (95% CI: 4-7 new cases per 100 pig-days) from 29-47 days of age and numerically decreased to 2 new cases per 100 pig-days (95% CI: 1-7 new cases per 100 pig-days) at 47-94 days of age. In contrast, in system B, as evidenced by the sharp increase in cases from 17-27 days of age (Figure 2), the incidence rate was 9 new cases per 100 pig-days (95% CI: 8-12 new cases per 100 pig-days) during that period.

4.4.2 Environmental samples

In each system, a total of 12 environmental samples were collected from farrowing crates. *M. hyorhinis* was not detected by qPCR in any of the 24 collected samples. All environmental samples were collected from litters where the piglet and sow tested negative in the nasal cavity.

4.4.3 Oral fluids

Detection of *M. hyorhinis* in oral fluid samples was consistent with the detection in the nasal cavity of the study pigs. In both systems, oral fluid samples were collected from

postweaning pigs at three different sampling points. In system A, 33% (3/9, 95% CI: 72-100%) of ropes tested positive at 29 days of age (Figure 4). In subsequent samplings all oral fluid samples tested positive by qPCR. In system B, all oral fluid samples collected from pigs at 27, 64 and 140 days (25/25) were positive by qPCR (Figure 4). The range of bacterial load from all oral fluids collected in system A was 4.1-5.6 $\log_{10}$ geq/mL and in system B was 4.2-7.6 $\log_{10}$ geq/mL.

4.4.4 Serology

Serum samples were collected from 107 and 111 sows and tagged piglets in the farrowing rooms of systems A and B respectively. Additionally, serum samples were collected from the same group of pigs at four subsequent sampling points (Figure 1). The complete distributions of serum titers for study pigs are displayed in figure 5. A total of 4/107 and 13/111 piglets at birth had no detectable serum antibodies measured by ELISA, in systems A and B, respectively. The median serum titer for piglets at birth was 25600 and 1600 for systems A and B respectively (Figure 5). At 17 days (weaning) the median titers decreased to 800 in system B. Median serum titers were 1600 at 47 and 64 days in system A and B, respectively. These values increased to 6400 and 3200 at 94 and 140 days in pigs from system A and B respectively (Figure 5). Sow median serum titer values are reported in Table 2.

4.4.5 Clinical signs and post-mortem observations

Typical clinical signs of polyserositis were not very frequent during the nursery peak of mortality of system A and none of the study pigs showed any clinical signs; therefore, only 8 pigs were selected for necropsy from the same cohort of the study pigs. Of those, 5/8 pigs had pericarditis. Of these, 2/5 tested positive for *M. hyorhinis* by qPCR of pericardium samples. All pigs were positive in the nasal cavity by qPCR. *M. hyorhinis* was cultured from 2/8 bronchial, 2/8 pericardial and 4/8 nasal swabs. *H. parasuis* was detected in the pericardium of 7/8 by PCR. A total of 3/3 pools of lung tissue were positive for IAV by PCR. Pigs tested negative for PRRSV, *M. hyopneumoniae* and PCV2. The main clinical sign observed in grow-finishing pigs was lameness and all 10 necropsied pigs had lesions of arthritis. A total of 8/10 tested positive for *M. hyorhinis* by qPCR in the joint sample, while 3/10 were also positive for *Mycoplasma hyosynoviae* by PCR. *M. hyorhinis* was also detected from 10/10 bronchial, pericardial and nasal swabs by qPCR and was isolated from 10/10 bronchial, 2/10 nasal, 2/10 pericardial and 0/10 joint swabs. All tissue pools tested positive for IAV and PRRSV. In addition to the 8 and 10 sick pigs necropsied during nursery and finishing periods, two healthy pigs were necropsied at each sampling time. *M. hyorhinis* was detected in the nasal cavity of all four healthy pigs and in 1/2 bronchial swabs from pigs in the finishing. However, *M. hyorhinis* was not detected in any systemic sites from healthy pigs. The veterinarian of this herd reported that this group of pigs experienced lameness until slaughter age. A total of 9 study pigs were sampled at the processing plant. All pigs had swollen joints and 6/9 had lymphoplasmacytic synovitis lesions observed by histopathology. *M hyorhinis* was

detected in 9/9 tonsils and 1/9 joint swabs. *M. hyosynoviae* was detected in 2/9 tonsils and 1/9 joint swabs by PCR.

In system B, most of the mortality was concentrated in the nursery. The most obvious clinical sign was lameness. Five out of ten necropsied pigs had arthritis and 4/5 tested positive for *M. hyorhinis* by qPCR of a joint sample. *M. hyorhinis* was cultured from 8/10 bronchial, 4/10 joint, 4/10 pericardial and 1/10 nasal swabs. *M. hyorhinis* was detected by qPCR in 5/10 pericardial, 6/10 nasal and 8/10 bronchial swabs. Diseased pigs tested positive for PRRSV, PCV2 and IAV by PCR and negative for *M. hyopneumoniae*. Finishing pigs did not experience a peak of mortality or disease. Pigs remained healthy throughout the finishing stage and therefore necropsies were not performed. Diagnostic submissions from sporadic deaths during the grow-finish phase of this group of pigs revealed pneumonia associated with PRRSV and *Actinobacillus suis* infection.

4.5 Discussion

The main objective of this study was to investigate the infection dynamics of *M. hyorhinis* in affected herds through a prospective longitudinal study. In general, this study confirmed results observed in chapter 3. The prevalence of *M. hyorhinis* infection

observed in piglets and sows was low (<5%). This observation is in accordance with findings of chapter 3 and with a previous study where *M. hyorhinis* was cultured from the nasal secretions of 7% (2/27) of sows (Ross and Spear 1973). The low prevalence detected here complements existing evidence reported for other swine mycoplasmas such as *M. hyopneumoniae* and *M. hyosynoviae*. Sibila et al. (2007) found 0-6.4% of piglets positive for *M. hyopneumoniae* prior to weaning (Sibila et al., 2007) Similarly, Fano et al. (2007) reported *M. hyopneumoniae* prevalence estimates of 0-51.2% in piglets from different cohorts at weaning (Fano et al., 2007). Ross and Spear (1973) isolated *M. hyosynoviae* from the nasal cavity of 7-14% of sows and 0.35% of piglets of 6 weeks of age (Ross and Spear, 1973). Contrary to what was observed in chapter three, where younger sows were more likely to be positive in the nasal cavity compared to older sows, in this study no significant difference was observed between the proportion of positive young and old sows. However, in system B only parity 1 sows where positive. One study carried out to evaluate nasal detection of *M. hyopneumoniae* found no statistical difference among parities in sows from parity 1 to 7 (Calsamiglia and Pijoan 2000). In the present study, it is possible that the difference in the proportion of *M. hyorhinis* PCR-positive sows between the young and old groups was smaller in these two herds that in the herds studied in chapter three, making the estimated sample size insufficient to detect the potential difference. It is also possible that a parity effect is only observed in some herds, potentially driven by gilt source or management. If there was a parity effect on *M. hyorhinis* infection and shedding, specific interventions at the breeding herd level, such

as parity segregation, could have an effect in the disease observed in the nursery and grow-finish phases.

The identification of the source of infection for suckling piglets is an important component in the understanding of disease epidemiology. Because the sows are in direct contact with their piglets in the farrowing room, they represent the most logical source of infection for piglets. However, in this study, infected piglets originated from PCR-negative sows and PCR-positive sows had negative piglets. In addition, no relationships were observed between sow parity and seropositivity with piglet nasal infection or seropositivity. Although a possible explanation is the inability of the diagnostic protocol to detect the presence of *M. hyorhinis* in the nasal cavity of the sows, this is unlikely given the small amount of microorganisms that the qPCR assay can detect (Clavijo et al., submitted 2014). However, it is possible that sows shed *M. hyorhinis* intermittently, which has been suggested as an occurring phenomenon with *M. hyopneumoniae* (Ruiz et al 2003) and *Mycoplasma bovis* (Maunsell et al 2011). Studies looking at the relationship between the infection in the sow and the piglet have been carried out for *M. hyopneumoniae,* with contradictory results. Fano et al (2006) found a higher proportion of positive piglets at weaning originating from young sows (p1-2) (Fano et al., 2006, while Sibila et al (2007) found no significant relationships between sow parity and piglet nasal infection and seropositivity (Sibila et al., 2007). Other possible source of infection for piglets could be through direct or indirect contact with older suckling piglets. Pigs in this study were not in direct contact with older piglets because the farrowing rooms were

managed AIAO. The indirect route was investigated by testing environmental samples from the farrowing rooms. Negative results for these environmental samples suggest that there is no indirect transmission, although it is possible that the low sample size hindered the ability to detect *M. hyorhinis* in the environment, especially under the low-prevalence scenario observed in these farms at the farrowing room. Therefore, the source of infection for suckling piglets could not be determined in this study.

In the previous chapter, due to the transversal study design, it could not be concluded that a rapid transmission occurred after placement in the nursery. In this study, as evidenced in both systems, over 90% of the pigs become colonized during the nursery phase. The incidence rate estimations demonstrated a relatively high number of pigs acquiring the microorganism within a short period of time. To the author's knowledge this is the first study to show that pigs can remain positive for most of the post-weaning phase, where the vast majority of pigs remained infected in the nasal cavity until 94 days in system A and 140 days in system B. Additionally, in system A, the tonsils from a subset of the study pigs swabbed at slaughter tested positive, indicating that a pig can remain positive until processing (~200 days of age). Interestingly, different clinical presentations and timing of disease were observed between systems. Research to identify herd-level risk factors for *M. hyorhinis* disease, as well as potential variability within a system between different cohorts is warranted.

Recently the testing of oral fluids has proven to be a very useful tool for pathogen monitoring and surveillance (Prickett et al., 2008). In this study the detection of *M.*

hyorhinis in oral fluid samples was consistent with the detection in the nasal cavity. Future studies are needed to determine the sensitivity of oral fluids for M. hyorhinis detection at the pen level (Romagosa et al., 2011; Ramirez et al., 2012; Olsen et al., 2013)

In this study, infected pigs had serum antibodies against *M. hyorhinis*. Since *M. hyorhinis* was detected in the nasal cavity of piglets with colostral-derived antibodies, it appears that maternal antibodies do not completely protect against *M. hyorhinis* nasal infection. Shortly after weaning these antibodies decreased which coincided with the increase in *M. hyorhinis* prevalence. During the peak of mortality in each system, it appears that there was an increase in serum titers, presumably due to development of acquired immunity. Future studies should evaluate the role of serum antibodies in protection against *M. hyorhinis* disease, as well as the detection of *M. hyorhinis*-specific mucosal antibodies and their impact on infection.

This study provides needed information on the infection dynamics of *M. hyorhinis* in affected herds. Because of the participation of two different systems, the large sample size and the longitudinal nature of this study, the information generated here is relevant and useful to understand the infection of this pathogen in commercial herds. This study provides a framework to understand the dynamics of infection of this pathogen that will be useful to design targeted control measures to prevent disease in affected herds.

Table 4.1 Description of swine production systems.

	System A	System B
Breeding herds		
Herd size-	4700	6000
Herd Location (state)	MN	MN
Production type	Farrow-to-wean	Farrow-to-wean
Gilt source/management	Internal multiplication	Internal multiplication
PRRS	Negative	Positive unstable
M. hyopneumoniae	Positive	Positive
Antibiotic treatment:		
Piglets	Birth: long acting penicillin, spot treatment penicillin and gentamicin	Draxxin at weaning
Sows	Short acting Penicillin	Spot treatment penicillin
Vaccine protocol:		
Piglets	Day 4- autogenous bacterial: *S. suis, H. parasuis, E. Rhusiopathiae*, A. suis and PCV2 Weaning: autogenous bacterial + *M. hyopneumoniae*,	Weaning: *M. hyopneumoniae*, *E. Rhusiopathiae, Salmonella sp and L. intracellularis,*
Sows	Influenza, autogenous bacterial, *Leptospira sp*, Parvovirus, *E. Rhusiopathiae*, *C. perfringens, E. coli,* PCV2 and *M. hyopneumoniae*	Influenza, *Leptospira sp*, Parvovirus, *E. Rhusiopathiae*
Weaning age	18-21 days	17-22
Pre-weaning mortality rate (%)	10-12	10-12
Nurseries		
Location	MN	MN
Production type	Wean-to-finish	Traditional nursery
Pig Flow	Multisource AIAO	Single source flow AIAO
Capacity/per site	4000	6000
Stocking density (m^2/animal)	0.3	0.3
Number of pigs per pen	50	25

Antibiotic treatments	Denagard/CTC (feed), amoxicillin and neomycin (water). Aspirin in water	Spot treatment penicillin, amoxicillin aspirin (water). Pulses of CTC (feed) and tetracycline (water)
Vaccination protocol	*M. hyopneumoniae*, PCV2, *E. Rhusiopathiae, Salmonella sp and L. intracellularis,*	PCV2, *M. hyopneumoniae*, *E. Rhusiopathiae*, *Salmonella sp and L. intracellularis,*
Mortality rate (%)	3	2-4
Finishers:		
Location	MN	MN
Production type	Multisource Grow-finish	Single source grow-finish
Pig Flow	AIAO	AIAO
Capacity/per site	2000	2300
Stocking density (m^2/animal)	0.7	0.7
Number of pigs per pen	25	25
Antibiotic treatments	CTC (feed), citric acid and aspirin (water)	First two weeks: Tiamulin/CTC (feed)

Figure 4.1 Prospective longitudinal study timeline.

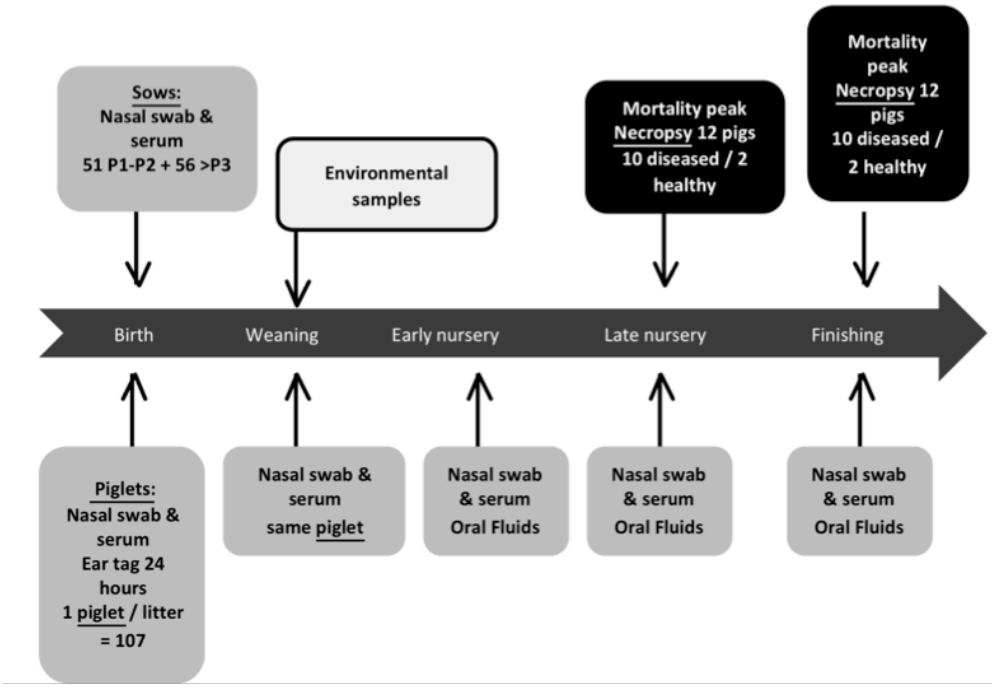


Figure 4.2 Prevalence of *M. hyorhinis* infection by pig age and 95% confidence interval.

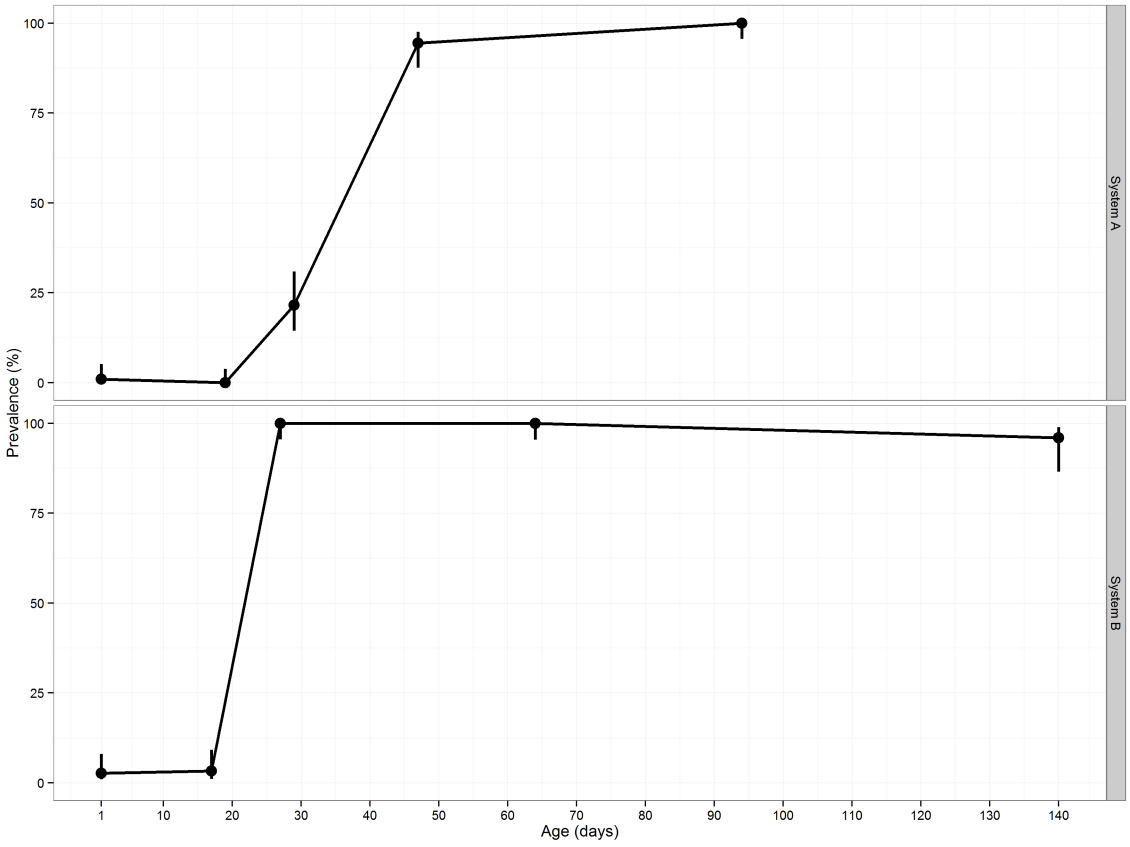


Table 4.2 Prevalence of *M. hyorhinis* infection, parity, bacterial load and serum titer in sows.

	System A	**System B**
Parity distribution (n)	1(17), 2(34), 3(18), 4(13), 5(7), 6 (7), 7(3), 8(2)	1(21), 2(33), 3(15), 4(9), 5(4), 6(11), 7(10), 8(7)
Prevalence	5/107	2/110
Bacterial load range ($\log_{10}$ geq/swab)	3.6-4.3	4.6-4.7
Median serum titer (range)	6400 (800-51200)	1600 (400-12800)

Figure 4.3 Bacterial load of *M. hyorhinis* in nasal cavity of pigs over time. The upper and lower hinges correspond to the 25th and 75th percentiles, respectively. The hinge within the box represents the median. Whiskers extend from each hinge to the highest and lowest value that are within 1.5 * the inter-quartile range. Data not covered by whiskers are outliers and are represented by points (McGill et al., 1978).

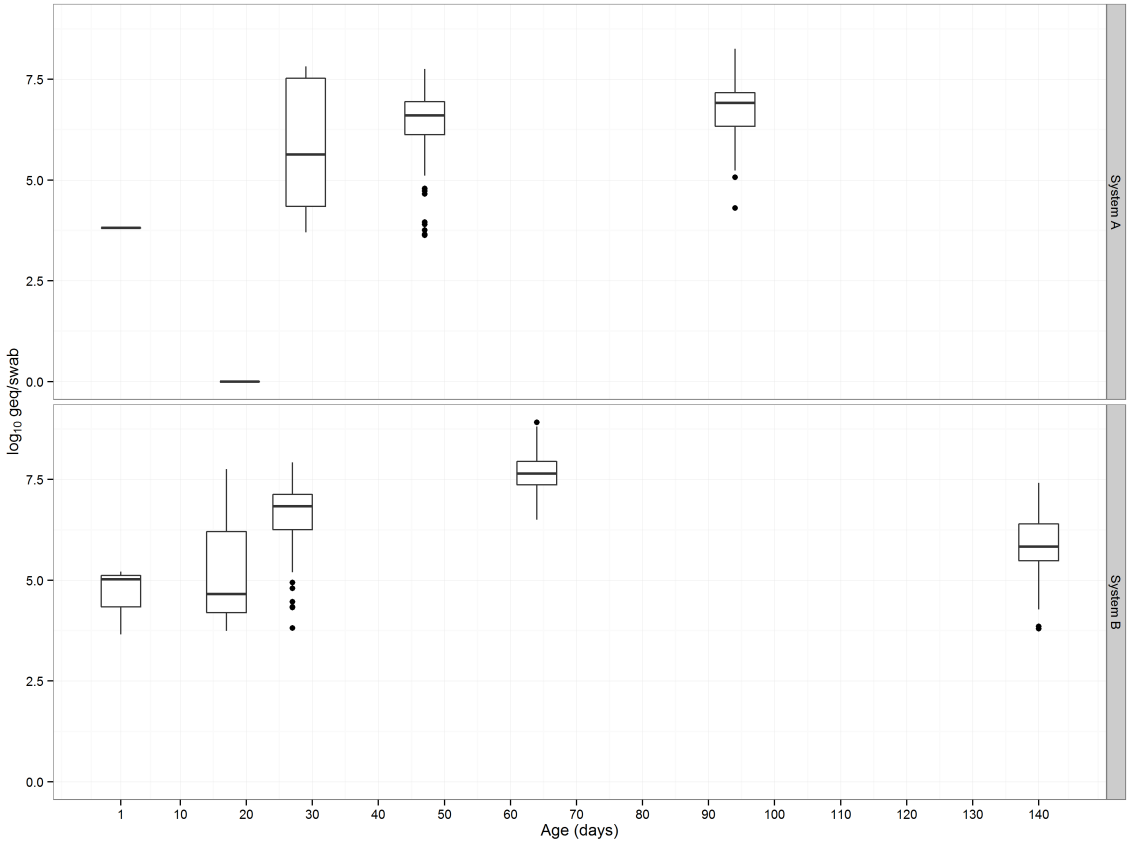


Figure 4.4 Proportion of *M. hyorhinis* positive nasal swabs and oral fluid samples. Error bars represent 95% confidence intervals.

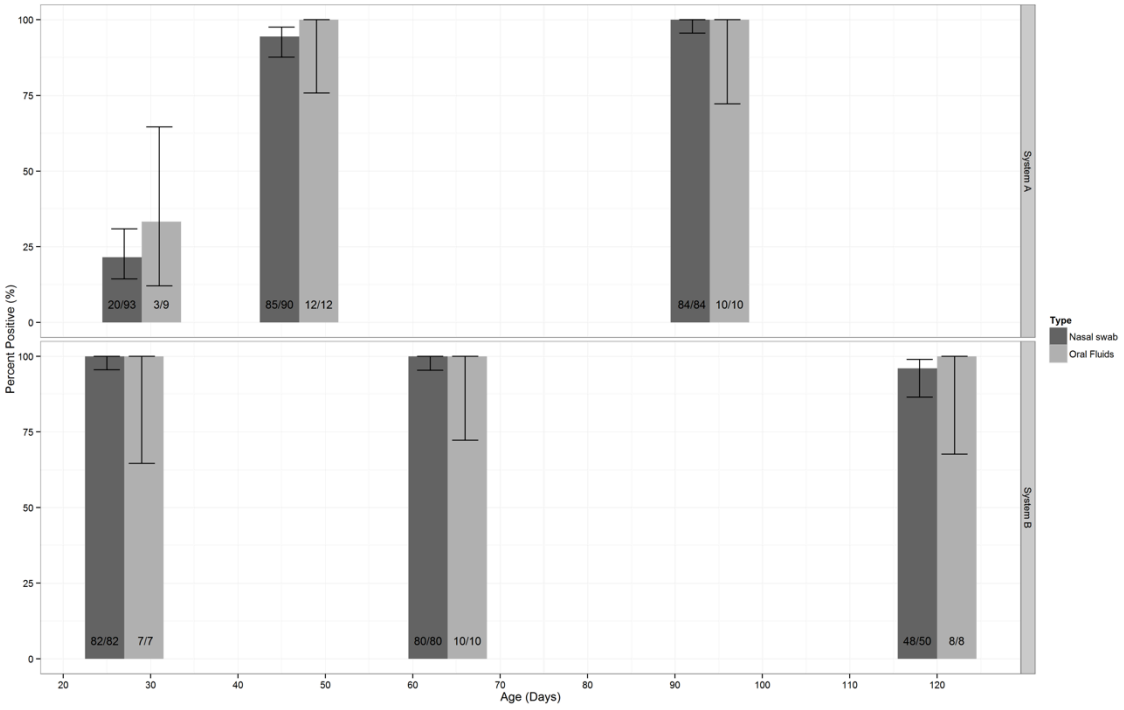
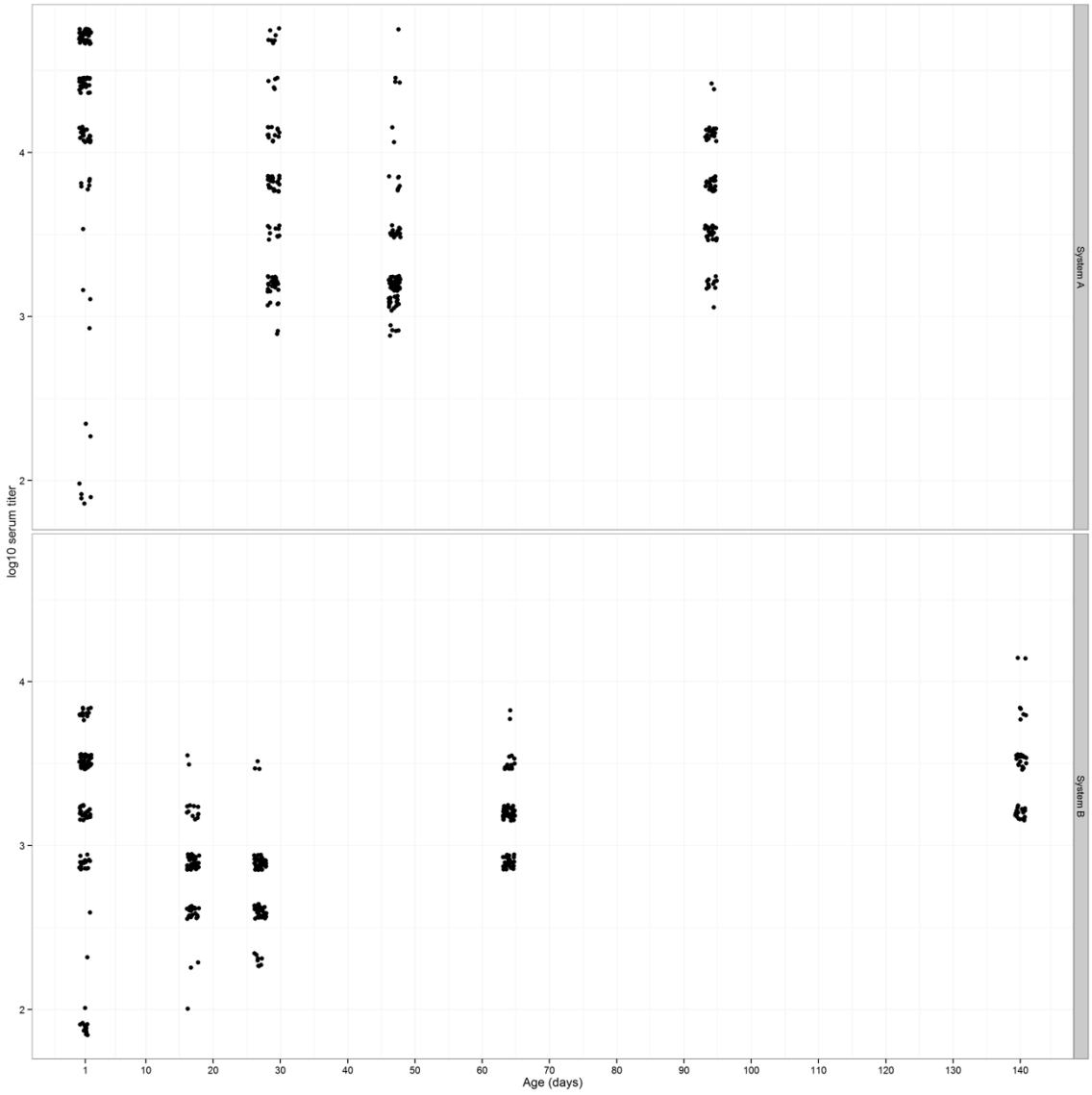


Figure 4.5 Serum antibody titers of pigs over time obtained by ELISA.



CHAPTER V
MOLECULAR EPIDEMIOLOGY OF *MYCOPLASMA HYORHINIS* U.S FIELD ISOLATES

Clavijo, MJ., Sreevatsan S., Johnson T., Rovira, A. Molecular epidemiology of *Mycoplasma hyorhinis* U.S field isolates. [in prep, to be submitted to Journal of Clinical Microbiology]

5.1 Summary

M. hyorhinis is one of the causative agents of polyserositis and arthritis in post-weaning pigs. It appears that differences in virulence of the infecting *M. hyorhinis* strain, the host immune response, and concomitant infections may play a role on disease manifestation. The objective of this study was to develop a multi-locus sequence typing (MLST-s) protocol for the characterization of *M. hyorhinis* field isolates. A total of 104 *M. hyorhinis* field isolates, mostly from the US, were utilized. A total of 23 genes, including housekeeping and surface encoding proteins, were evaluated as target genes to index diversity. Genes encoding surface proteins were included to increase the discriminatory power of the MLST, which is important for epidemiological investigations. Genes where discarded when the sequences of *M. hyorhinis* HUB-1, GDL-1, SK76 and MCLD where identical, when nucleotide variability restricted primer placement and when no PCR amplification product was obtained or had a poor reproducibility. Four target genes were selected to be included in the final MLST-s protocol: *pdhB*, *p95, mtlD* and *ung*. Within each locus the nucleotide variation ranged from 1.4% to 20%. The 104 filed isolates were classified in 39 different sequence types (STs). Multiple STs were found within the same production system and even within the same pig. The majority of STs grouped strains from the same production system, but there were cases where multiple systems shared the same ST, indicating a potential common source of pig flow. The majority of the nucleotide changes observed generated synonymous changes, while non-synonymous changes were exclusively in the *mtlD* gene fragment. Molecular typing of *M. hyorhinis*

will aid in better understanding transmission routes, in assessing sources of infection and also in evaluating interventions such as vaccination and use of antibiotics.

5.2 Introduction:

In recent years *Mycoplasma hyorhinis* has been recognized as an important cause of mortality in nursery pigs (Leuwerke, 2009; Rovira et al., 2010). This pathogen is usually found in the upper respiratory tract of colonized pigs, more specifically in nasal secretions and in the oropharyngeal surface. Piglets presumably become colonized by contact with the sows and the bacterium is transmitted through nose-to-nose contact among pigs afterwards (Kobisch and Friis, 1996). Although *M. hyorhinis* infections are frequently subclinical, it can cause polyserositis and arthritis in postweaning pigs. In fact, 55% of polyserositis and 12% of arthritis cases received at the Minnesota Veterinary Diagnostic Laboratory tested positive for this pathogen by PCR (Rovira et al., 2010). *M. hyorhinis* associated disease in finishing stage pigs is usually characterized by arthritis only (Gomes-Neto et al., 2011). Other clinical presentations including pneumonia, otitis and conjunctivitis have been reported for this organism (Gois et al., 1971; Friis, 1976; Morita et al., 1999). However, the role of *M. hyorhinis* in these disease presentations is uncertain.

While this pathogen was first described in 1955, very little research has been generated regarding the ecology and epidemiology of this organism, which is needed in order to design effective control and prevention protocols. In chapter three, the prevalence of *M. hyorhinis* infection in three different herds was estimated. Interestingly,

even though the three herds were managed under very similar conditions, two very different patterns of colonization and clinical presentation were observed. In two of these herds, most pigs became colonized with *M. hyorhinis* at the beginning of the nursery and *M. hyorhinis* was the main cause of nursery mortality. In contrast, pigs in herd C did not become colonized until the last week of the nursery period and did not suffer from *M. hyorhinis* associated disease. One possible explanation for these different temporal patterns of infection and disease manifestation could be the presence of different *M. hyorhinis* strains with different infection and virulence capacities. In this manuscript strain is defined as an isolate or group of isolates that share identical phenotypic or genetic characteristics and sets them apart from other isolates from the same genus (Tenover et al., 1995; Zadoks and Schukken 2006)

There are several pieces of information in the literature that indicate that there is heterogeneity within the *M. hyorhinis* species. Antigenic differences between different *M. hyorhinis* isolates have been shown by seroreactivity to specific antisera (Friis, 1971). This antigenic variation might be determined in part by the presence of a highly complex system of variable lipoprotein expression, which allows for great surface variation (Rosengarten et al., 1991). In addition to this antigenic variation, several experimental challenge studies have shown differences in virulence *in vivo* (Shulman et al., 1970; Gois et al., 1971; Gois M. & Kuksa F. 1974; Kinne et al., 1991). Furthermore, the clinical

presentation of disease caused by *M. hyorhinis* varies from herd to herd. While some herds are repeatedly affected by *M. hyorhinis*-associated arthritis, others tend to see mostly polyserositis (personal observations of Dr. Rovira). Likewise, in chapter four, differences in timing of clinical disease were seen within and between systems. In system A, the disease observed in the nursery was polyserositis, while arthritis was the only *M. hyorhinis*-associated disease observed in finishing pigs. In system B, disease was only evident in the nursery, where pigs suffered arthritis. This diverse disease presentation also suggests that there may be strains with different tissue affinities.

At the genome level, the use of pulsed-field gel electrophoresis (PFGE) to index variation within the *M. hyorhinis* species has revealed genetic heterogeneity within the species (Barlev and Borchsenius 1991; Ling and Weng 2000). However, PFGE is time consuming and interpretation of the banding patterns in some cases can be ambiguous. Furthermore, since it identifies major genetic changes, small DNA changes will be missed (Tenover et al., 1995; Lau et al., 2000). Sequence based tools, such as MLST (multilocus sequence typing), have been widely used for swine bacterial genotyping (King et al., 2002; Olvera et al., 2006; Mayor et al., 2008). Furthermore, MLST is considered a rapid, inexpensive, portable and unambiguous typing tool (Maiden et al., 1998). The classical MLST protocol is based on the sequencing of approximately 500 base pairs of multiple housekeeping genes (core metabolic genes) for unambiguous characterization of bacterial isolates. These genes tend to slowly accumulate variation,

providing the foundation for long-term epidemiological and evolutionary biology studies. Distinct nucleotide sequences within each gene are defined as different allele numbers and the combination of these for each of the loci gives rise to the allelic profile, also referred as sequence type (ST) (Maiden et al 1998). Recently, an MLST based on 6 housekeeping genes revealed genetic variation between 33 field isolates of *M. hyorhinis* originating from pigs in different regions of France. The 33 isolates were classified into 29 distinct sequence types (STs) (Tocqueville et al., 2014). However, the authors did not assess the use of hyper-variable loci (i.e. surface-associated proteins, adhesins) that could provide a higher resolution, and could therefore be more informative in outbreak investigations (Cooper and Feil, 2004).

In this manuscript we describe the development of a MLST assay based on housekeeping and surface encoding proteins to differentiate *M. hyorhinis* strains, termed MLST-s to reference the addition of surface proteins. The addition of surface proteins was done with the objective of generating a increased discriminatory power since these genes may be subject to more intensive selective pressures, compared to housekeeping genes, making them ideal for local epidemiology studies (Cooper and Feil 2004; Tankouo-Sandjong et al., 2007).

5.3 Materials and methods

5.3.1 Bacterial isolates, culture media and DNA extraction

A total of 121 *M. hyorhinis* isolates from pigs along with an ATCC reference strain (17981D) were studied. One hundred and four isolates originated from the nasal cavity (n=10), bronchus (n=26), pericardium (n=26), pleura (n=21), peritoneum (n=2) and joint (n=19) of diseased pigs with lesions of polyserositis and a clinical history suggestive of *M. hyorhinis* infection (fever, depression, lameness, dyspnea). Seventeen isolates originated from the bronchus (n=2) and nasal cavity (n=15) of healthy pigs. Finally, one isolate was obtained from an air sample taken from the outside of a barn housing nursery pigs. All isolates were obtained from cases submitted to the UMN Veterinary Diagnostic Laboratory from 2010 to 2012. The isolates originated from multiple farms (nurseries, finishers and gilt acclimation sites) belonging to 22 different swine production systems (denoted as numbers 1-22) in 11 states. Isolates were stored at -80 °C until further testing, for which they were re-grown in 3 mL aliquots of modified Hayflick's medium for 2 to 14 days (Lefebvre et al., 1987). The suspension was centrifuged and *M. hyorhinis* genomic DNA was extracted using the DNeasy Blood & Tissue kit, following the manufacturer's protocol (Qiagen, Germantown, MD).

5.3.2 Loci selection and primer design

The whole genomes of four *M. hyorhinis* isolates available in GenBank (HUB-1 (NC_014448.1), GDL-1 (NC_016829.1) MCLD (NC_017519.1) and SK76

(NC_019552.1)) were utilized to identify potential target genes. Identification of variable regions within the *M. hyorhinis* species was accomplished through a Clustal W progressive alignment of all 4 *M. hyorhinis* genomes using Mauve 2.3.1 (Darling et al., 2010). Further identification of areas of variation within different housekeeping and surface protein encoding genes, such as previously identified variable lipoproteins and adhesins previously identified (Siqueira et al., 2013), was performed using Geneious 7.0.6 (Biomatters, Auckland, New Zealand, http://www.geneious.com). Primers were designed using PrimerQuest® (IDT, Coralville, USA) software. Genes with observed genetic variation between the available *M. hyorhinis* genomes, were further tested with a subset of 6 isolates with different epidemiological background. Candidate genes were discarded if no nucleotide sequence variation was observed between these 6.

5.3.3 PCR conditions and sequencing

All qPCR reactions were prepared in a volume of 25 µl consisting of 2 µl of the extracted DNA, 12 µl of Master Mix solution (HotStarTaq Master Mix, Qiagen, Germantown, MD), 0.8 µl of each 10 µM reverse and forward primer and 9.4 µl of DNAse free H_20 (Table 1). Target genes where PCR amplified using an initial denaturation step at 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 43.5 °C for 1 min 30 sec. Determination of an optimal annealing temperature was accomplished by gradient pcr. Electrophoresis on a 1% agarose gel was performed on the amplified products and bands were observed under ultraviolet light in the presence of ethidium bromide. PCR products

were purified using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD) and bi-directionally sequenced by standard Sanger sequencing on an AABI 3730xl BigDye Terminator v 3.1 (Life Technologies, Grand Island, NY).

5.3.4 Data analysis

Resulting sequencing data was quality assessed and sequences were aligned utilizing ClustalW within Geneious 7.0.6 and trimmed to equal sizes. MEGA 5.2.1 (version 5; www.megasoftware.net) was utilized to identify parsimony-informative sites. Nucleotide sequence alignments for each gene fragment where translated using the mycoplasma genetic code to assess potential synonymous and non-synonymous substitutions. Individual and concatenated dendrograms were computed using the Hasegawa-Kishino-Yano distance model and the UPGMA (unweighted pair group method with arithmetic mean) tree building method. To define the possible contribution of mutation and recombination the standardized index of association (I_A^s) was calculated using the LIAN program (Haubold and Hudson, 2000, http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl). Briefly, this software tests the null hypothesis of linkage equilibrium between the different MLST loci, using a Monte Carlo simulation and an algebraic method. $I_{A,}$ represents a function of the rate of recombination, a value of zero for I_A indicates linkage equilibrium, showing that recombination events must occur frequently. A statistically significant deviation from zero signifies linkage disequilibrium, indicating that recombination should be rare.

For each targeted locus, each distinct sequence variant was assigned a consecutive number starting with 1, with no weight given to the degree of nucleotide sequence variation among alleles (Maiden et al., 1998). A distinct sequence type (ST) or allelic profile number was defined for each combination of the allele numbers for each locus (i.e. 1-5-2-3). The STs were assigned consecutive numbers in order of description (i.e. ST1). Relationships among strains were evaluated using minimum spanning trees (MST) analysis, after the introduction of allelic profiles into Bionumerics software V7.1 (Applied Math, Sint Maartens-Latem, Belgium). This method groups strains into clonal complexes, defined here as "as a group of MLST genotypes in which every genotype shares at least 3 out of 4 loci in common with at least one other member of the group" (pubmlst.org/analysis/burst/burst.shtml).

5.4 Results

5.4.1 Selection and evaluation of *M. hyorhinis* MLST-sp marker genes

A total of 23 genes were evaluated as potential target genes. These included housekeeping genes: beta-phosphoglucomutase *(pgmB),* translation elongation factor G *(fusA),* DNA gyrase subunit B *(gyrB),* GTP-binding protein *(lepA),* methionine tRNA

ligase *(metS),* glutamyl-tRNA synthetase *(gltX),* chromosomal replication initiator protein *(dnaA),* uracil-DNA glycosylase (*ung*), and pyruvate dehydrogenase E1 component beta subunit (*pdhB)*; adhesin encoding genes: *p3, p95, p37,* predicted surface protein encoding genes: mannitol-1-phosphate 5-dehydrogenase (*mtlD*), hexosephosphate transport protein (*hexo*), ribonucleoside-diphosphate reductase (*nrdf*), lipoprotein Signal peptidase II (*lspA*), and cardiolipin synthetase (*cls*) and variable lipoproteins (*vlp*) *A,B,C,D,E,F* and *G*.

All housekeeping genes, but *pdhB* and *ung,* were identical in all 4 annotated genomes, therefore were not included for further testing. While variation was observed within the *nrdf* and *lspA* genes from the 4 annotated genomes, further testing with the 6 *M. hyorhinis* isolates revealed no nucleotide sequence variation and were therefore not included for further testing. On the contrary, the gene sequences for *vlpF* and *B* genes were found to be highly divergent among the four available genomes, with no conserved flanking regions, hindering the possibility of primer placement. Primers for the remaining *vlp* genes were designed, however, an amplicon was not obtained for *vlpA, C, D* and *E* gene when testing a subset of *M. hyorhinis* isolates (n=6). Although and amplicon was generated for the *vlpG* gene, the generated product had low quality and was not reproducible. A total of 8 target genes were utilized to further test isolates: *cls*, *hexo, p3, p37, p95, pdhB*, *ung*, *mtlD*. Nucleotide sequence variation was observed within the *cls*, *hexo* and *p37* genes when preliminary testing was carried out on 6 isolates. Further testing of these 3 genes on 39 isolates with different epidemiological background

revealed minimal genetic variation between isolates and where therefore excluded from the subsequent testing of the remaining isolates (n=64). The discriminatory power of the technique was not affected after the removal of the three genes (*p37*, *cls* and *hex)* as evidenced when analyzing the initial 39 isolates and STs obtained (data not shown). The 64 remaining isolates originated from the same systems and during the same or similar time frames as those from the first 39 isolates. A poor sequencing quality was obtained from the remaining isolates (n=64) with the *p3* gene and thus was excluded from further analysis. The final MLST-s protocol included: *pdhB*, *p95*, *mtlD* and *ung* (Table 1), which were tested. The selected genes were dispersed throughout the *M. hyorhinis* genome, as observed in the circular DNA map created using DNAPlotter software (Carver et al., 2009) (Figure 1).

5.4.2 Relatedness of *M. hyorhinis* isolates

Evaluation of the chromatograms revealed a low quality of the sequencing data obtained for 17/121 isolates, therefore 104 isolates were utilized for the final analysis. The excluded isolates originated from the nasal cavity of pigs. The dendrogram constructed based upon concatenated gene sequences (1441bp) revealed genetic polymorphisms among the examined isolates (Figure 2). The nucleotide sequence variation (percent informative sites) within each gene ranged from 1.4 to 21% (Table 2), with *mtlD* showing the highest degree of variation and *pdhB* the lowest. Although nucleotide polymorphisms where observed, the majority resulted in synonymous changes in the *pdhB*, *p95* and *ung*

genes. In contrast, non-synonymous changes were seen within the *mtlD* gene (Figure 3). Furthermore, in some instances changes involved the switch from amino acids belonging to different groups, based on the characteristics of the side chain, polarity or acid-base properties (i.e. alanine to serine, glutamic acid to lysine) (Figure 3). The number of alleles per gene varied from 6 to 20, giving rise to 39 sequence types (STs) within the 104 isolates (Tables 2 and 3).

5.4.3 Evidence of recombination

The standardized index of association (I_A^s), which serves as a function for the rate of recombination, was estimated using one isolate per ST. The LIAN software gave a I_A^s of 0.0387 and was not significantly different from zero (P-value 0.07), indicating that the genetic variation observed was mostly due to recombination and not mutation events and that the genetic variation observed is in linkage equilibrium.

5.4.4 Frequency and distribution of *M. hyorhinis* sequence types (ST)

The most frequent ST, ST37, was observed 13 times. Other 18 STs contained between 2 and 9 isolates. Finally, 21 STs contained only one isolate. Minimum spanning tree analysis showed the grouping of 39 STs into 3 clonal complexes (CC), with CC1 encompassing more STs (n=25) and isolates (n=66) than any other clonal complex. CC 2 and 3 contained 2 STs each and a total of 2, and 6 isolates, respectively. Lastly,

singletons, defined as isolates which share less than 3 out of 4 loci with another member of a clonal complex, were identified 10 times and included 30 isolates (Figure 5).

ST37, the most frequent ST, and ST19 contained isolates from one system each, collected the same day during a *M. hyorhinis* outbreak investigation (Table 3). Furthermore, two isolates from the upper respiratory tract (nasal cavity or bronchus) and pericardium were obtained from the same pig, evidencing that isolates recovered from systemic sites of disease pigs can be also found in the nasal cavity or bronchi. ST19 was represented by isolates of healthy (nasal cavity or bronchi) and diseased pigs (joints) originating from system 2 in 2010 through 2012. However, isolates with different STs (ST18 and ST36) were also obtained from pigs from that system during the same time frame (Table 3). Furthermore, in system 2, isolates from the pleura and bronchus of the same pig had different STs, differing by only one allele (*mltD*). In contrast, in system 5 isolates from pigs in different states shared the same ST.

The isolates from ST17 (n=9) originated from systemic sites of pigs of a wide distribution of age (7-50 weeks), from 5 different systems in 4 different states in 2010-2012 (Table 3). It is important to note that information gathered for each isolate included system with refers to the ownership of the pigs but it lacked specific information on the pig flow or site, which might limit the epidemiological interpretation of the data

presented here. Since one system can be comprised of multiple producers, that submit cases independently, thus identifying them as different systems while the source of the pig was identical.

The reference strain HUB-1, originating from China, as well as the strains originating from Mexico showed a unique ST. CC2 and 3 were comprised by isolates from the same system each (Table 3). Finally, no evident relationships were observed with respects to the age of the pig, lesion type, year or state (Figure 5).

5.5 Discussion

The primary goals of molecular epidemiology are to identify sources of infection, to track the spread of infectious microorganisms and to evaluate persistence or reintroduction of a pathogen in a population. Moreover, molecular epidemiology can play an important role in disease control, since molecular methods can be used to determine potential vaccine targets as well as predicting vaccine efficacy (Zadoks and Schukken, 2006). The objective of this study was to develop a MLST-s typing scheme for epidemiological and genetic characterization of *M. hyorhinis* field isolates. More than 20 loci were evaluated as potential gene targets. However, many of these were almost identical in all four previously published *M. hyorhinis* genomes, and were therefore not pursued further. In view of this limited variation it was decided to incorporate more variable genes, such as

surface protein encoding genes. This modification could increase the possibility of detecting more genetic variation, making it useful for typing during localized disease outbreaks and studying the pathogenesis of *M. hyorhinis.*

Variable lipoproteins revealed an immense amount of variation between the available genomes, so much so that they lacked conserved areas for primer placing. These findings were somewhat anticipated given the fact that the genes coding for these lipoprotein, are under constant change, either through recombination or high-frequency mutations, affecting their expression or size (Citti and Wise, 1995; Citti and Blanchard, 2013)

This study demonstrated genetic variation within *M. hyorhinis* strains circulating in US swine production systems. The final MLST-s protocol contained 2 core or housekeeping genes, *pdhB* and *ung,* with an observed variation between 1.4-2% and 6-7 different allele types, respectively. Similar amounts of variation were observed within housekeeping genes in previously published literature. A recently developed MLST showed variation ranging from 0.9 to 2.1% within *M. hyorhinis* housekeeping genes (Tocqueville et al., 2014) Likewise, such genes incorporated to MLST schemes for *M. agalactiae*, *M. bovis*, and *M. hyopneumoniae* revealed genetic variability averaging 3%. On the other hand, the surface protein mtlD showed great variation (~ 21%) within the analyzed sequences and more than 20 different alleles. One unanticipated finding was the

little to no variation observed within widely recognized adhesins, such as p37 and p95. One possible explanation is that these proteins may be essential for host colonization and survivability (Razin et al., 1998). In fact it has been shown that truncated versions of the p95 adhesin contribute to loss of virulence in *M. hyopneumoniae* (Liu et al., 2012).

Even though variation was observed within *pdhB*, *p95* and the *ung* loci, these nucleotide changes resulted in mostly synonymous changes. In contrast, the amino acid sequence alignment of the mtlD gene revealed multiple non-synonymous changes. In some cases, the change was between amino acids of different chemical groups. Further analysis should evaluate if these changes influence the antigenic variation of this species or if it contributes to virulence since it is found in the bacterial surface.

Modified MLST protocols are not novel. Hyper-variable genes have been incorporated into typing schemes for *M. agalactiae*, *M. capricolum* and *B. pertussis* (Manso-Silvan et al., 2011; Manso-Silvan et al., 2012; van Loo et al., 2002). In all cases an increase in discriminatory power was obtained and in some cases this modification led to the identification of MLST profiles associated with epidemics and globally distributed strains (van Loo et al., 2002). The increased ability to discriminate between isolates during an epidemic or outbreak investigation might be at the cost of the interpretation of observed evolutionary relationships (Cooper and Feil 2004). In this study there was evidence of homologous recombination within the allelic dataset by estimating the

standardized index of association as a proxy for the rate recombination. Future studies should evaluate the potential effect of recombination on the observed phylogenetic relationships using this MLTS-s protocol.

The present study showed evidence of multiple STs circulating within US swine herds. Multiple STs were found within a system, during the same time frame and within diseased pigs. No correlation between STs and the age of the pig, the year of isolation, the sample type and state of origin was observed. Nonetheless, a clear relationship was observed between systems and STs. An over-representation of samples from diseased pigs could have hindered the identification of virulence associated STs. Although repeatability of the assay was not measured, a total of 7 isolates with different epidemiological background were re-tested and were identified as having the same ST as previously defined (data not shown)

The MLST-s scheme presented here was shown to be highly discriminatory, capable of subdividing 104 isolates into 39 distinct STs. ST17 included isolates from 5 different production systems. This could indicate the wide spread of a clone that may be a more successful pathogen or simply an organism with a longer persistence and survivability. However, it could also be due to the convenience nature of the sample

(McAuliffe et al., 2011). In contrast to the observed sharing ST17 between systems, the majority of the STs that had more than one isolate originated from one system.

Currently swine practitioners are faced with the challenge of controlling *M. hyorhinis* disease in affected post-weaning pigs. The application of the MLST-s protocol described here will result in a better understanding of the diversity of *M. hyorhinis* field isolates circulating in U.S swine herds. This tool will allow studying the epidemiology and dynamics of infection for this pathogen, understanding disease outbreaks, selection of isolates for vaccine production, and identification of the potential origin of a specific isolate.

Table 5.1 Primer sequences employed in MLST-s *M. hyorhinis* assay.

Target gene	Gene name	Forward (5'-3')	Reverse (5'-3')
pdhB	Pyruvate dehydrogenase-E beta-1 subunit	AACGACCCAGATCCAGTTATTTT	TCGTTAATTGCGTGGAATCCTTC
p95	Outer membrane protein p95	GTTGCCAAGCAAGATGCTAAA	TGCTGAATGTACTCACCTGAAA
mtlD	Mannitol-1-phosphate 5-dehydrogenase	GGTGCAGGTAGCATTGGTCGTGG	GCCAAGATAACCCAAATAAGAATGC
ung	Uracil-dna glycosylase	GCAAGAACAAGACAAAGAAT	TAGCTAAACGGTGAAGGATGAGATA

Figure 5.1 Circular representation of *M. hyorhinis* HUB-1 chromosome and location of targeted genes. The outer circle depicts scale in base pairs. Circles 2-3 indicate coding regions. Circle 4 depicts G+C content relative to the genome average of 26% (purple=below average, green=above average). Purple= *pdhB*, dark green= *p95*, black= *p3*, pink= *hex*, blue= *cls*, light green= *ung*, red= *mtlD*

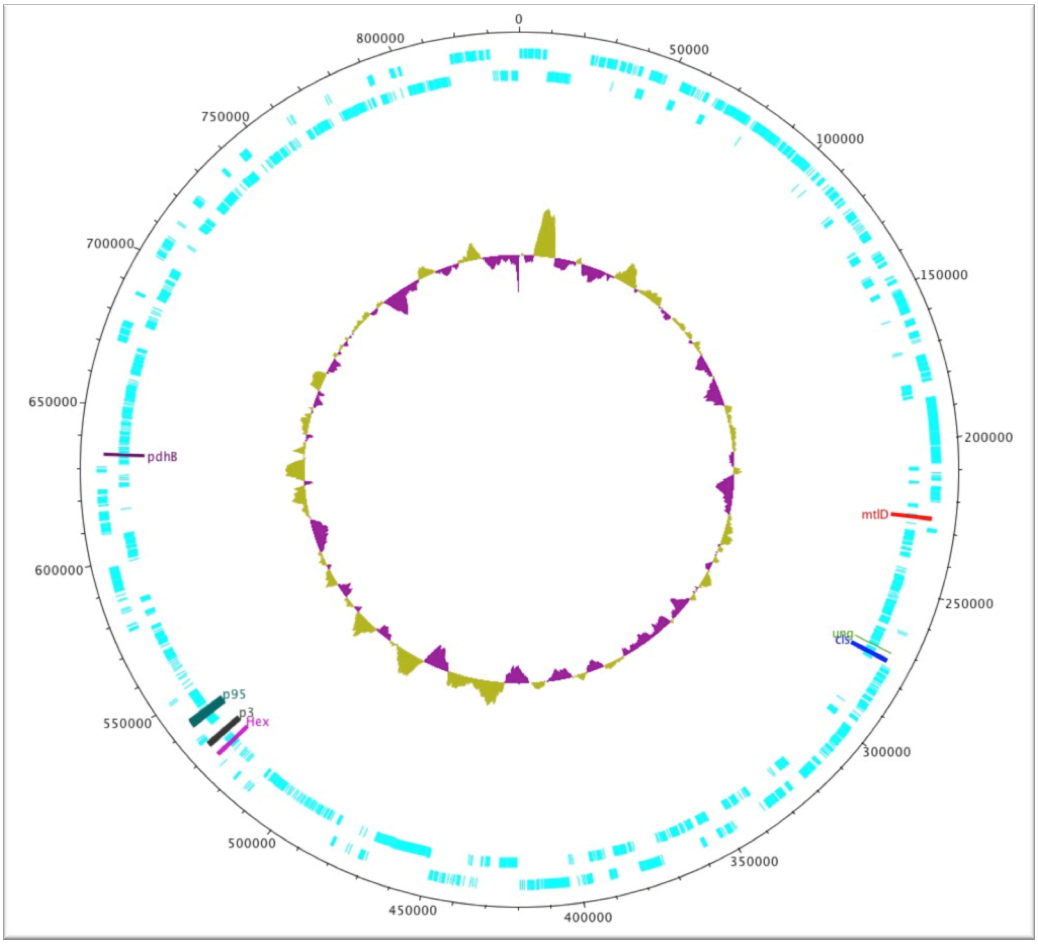


Table 5.2 Characteristics of regions used for multilocus sequence analysis and number of alleles

Target gene	Size (bp)	Trimmed length (bp)	Percent evaluated	Variable sites	Informative sites	Percent informative sites	No. of alleles
pdhB	987	415	42	6	6	1.4	7
p95	3171	289	9	8	6	2	9
mtlD	1101	537	49	120	115	21	20
ung	675	200	30	4	4	2	6

Table 5.3 Frequency and distribution of 39 *M. hyorhinis* STs within sample type, system, origin and age of pig.

ST	ST profile[a]	Frequency	Clonal complex	Sample type (no.)[b]	System[c]	State of origin	Age (weeks)
1	1-1-1-1	3	1	Pericardium	1, 21	MN	7,13
2	1-1-1-2	1	1	Pericardium	4	NC	3
3	1-1-1-3	2	1	Pericardium, pleura	8	MN	7
4	1-1-2-2	2	1	Pleura, joint	13, 14	TN, IL	7, 9
5	1-1-2-3	1	1	Pericardium	8	IA	9
6	1-1-3-2	1	1	Bronchus	9	NE	13
7	1-1-8-2	2	1	Joint	13	IL	9
8	1-1-11-2	1	1	Pleura	17	NC	NA
9	1-1-16-2	1	1	Bronchus	6	NE	5
10	1-1-17-2	3	1	Peritoneum (1), bronchus (2)	5, 22	OK, NE	4,11
11	1-3-1-1	1	1	Joint	12	MN	8
12	1-3-1-2	1	1	Pericardium	7	MN	11
13	1-3-5-4	1	S	Pleura	11	NC	10
14	1-4-3-2	1	1	Pleura	9	NE	13
15	1-5-1-1	4	1	Bronchus (1), pericardial (2), nasal cavity (1)	2	MN	8,11,13
16	1-5-1-2	1	1	Joint	2	MN	7
17	1-5-1-4	9	1	Bronchus (3), joint (1), pericardium (2), pleura (3)	5,7,10, 15,18	KS,MN,IN, OK	7,8,9,11 ,13,50
18	1-5-4-1	5	1	Bronchus(3), joint (1), nasal cavity (1)	2	MN	8,13
19	1-5-9-1	9	1	Bronchus (2), joint (3) nasal cavity (4)	2	MN	6,8,13
20	1-5-13-1	1	1	Pleura	2	MN	8
21	1-6-10-2	1	S	Pleura	16	PA	4
22	1-7-18-1	1	S	Pericardium	19	MEXICO	20
23	1-8-1-1	1	1	Pleura	12	MN	5
24	1-8-15-2	1	2	Pericardium	19	MEXICO	8
25	1-8-19-2	1	2	Pericardium	19	MEXICO	10
26	2-1-1-2	1	1	Nasal cavity	REF[d]	IA	NA

27	2-2-2-2	4	3	Pleura	5	KS, OK	3,6
28	2-2-7-2	2	3	Joint	NA	NA	NA
29	3-5-1-4	5	1	Bronchus, pericardium, pleura	5	OK	8,10
30	3-5-14-1	2	S	Aerosol, bronchus	8	MN	6
31	4-3-14-5	5	S	Pericardium	5	OK	8
32	4-5-15-2	6	S	Nasal cavity, pleura	5	OK	4,5
33	5-5-4-1	1	1	Nasal cavity	2	MN	8
34	5-5-4-2	1	1	Pericardium	2	MN	6
35	5-5-9-1	8	1	Bronchus (2), joint (3), pericardial (2), pleura (1)	2	MN	8
36	6-1-6-2	2	S	Joint	NA	NA	NA
37	7-3-12-2	13	S	Bronchus (8), nasal cavity (3), pericardium (2)	7	MN	14
38	7-8-3-2	1	S	Pericardium	20	MN	10
39	7-9-20-6	1	S	Nasal cavity	HUB-1[e]	CHINA	NA

[a] Order of allele number for each locus: *pdhB*, *p95*, *mtlD* and *ung*
[b] Type of sample where isolate was obtained
[c] Systems are defined as the same owner and denoted as a number from 1-22
NA- not available
S- singleton, not part of a clonal complex
[d] Reference strain (ATCC 17981)
[e] HUB-1 reference genome (GenBank)

Figures 5.2 Inferred relationships between 104 *M. hyorhinis* isolates using concatenated sequences for genes: *pdhB*, *p95*, *mtlD* and *ung.* Data was analyzed in Geneious using the UPGMA tree method based on the Hasegawa-Kishino-Yano model. A total of 1,441 positions were employed in the final dataset and 1500 bootstrap replicates. Scale represents nucleotide substitutions per site.

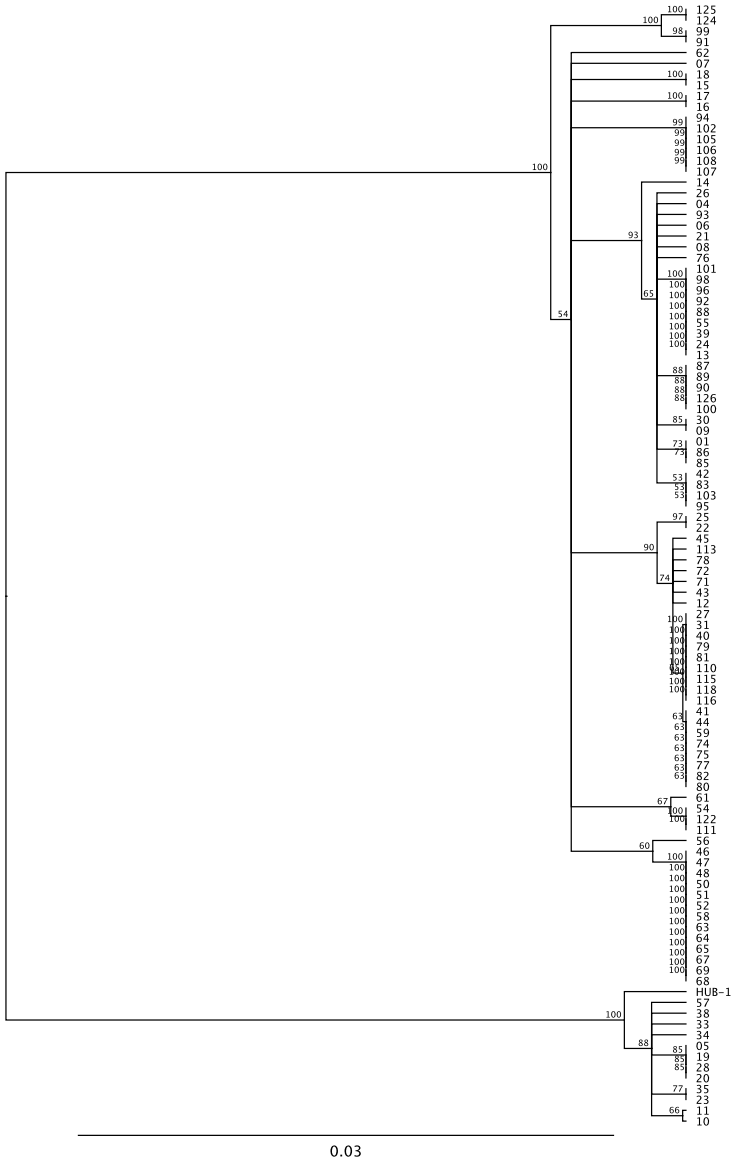


Figure 5.3 Amino acid sequence alignment for the *mtlD* gene. Arrows indicate areas of non-synonymous changes between aligned sequences where the amino acid change was between different amino acid groups, based on polarity, pH level or side chain. Arrows from left to right: lysine (basic) vs. glutamic acid (acid), alanine (non-polar) vs. serine (polar) phenylalanine (aliphatic group) vs. leucine (aromatic).

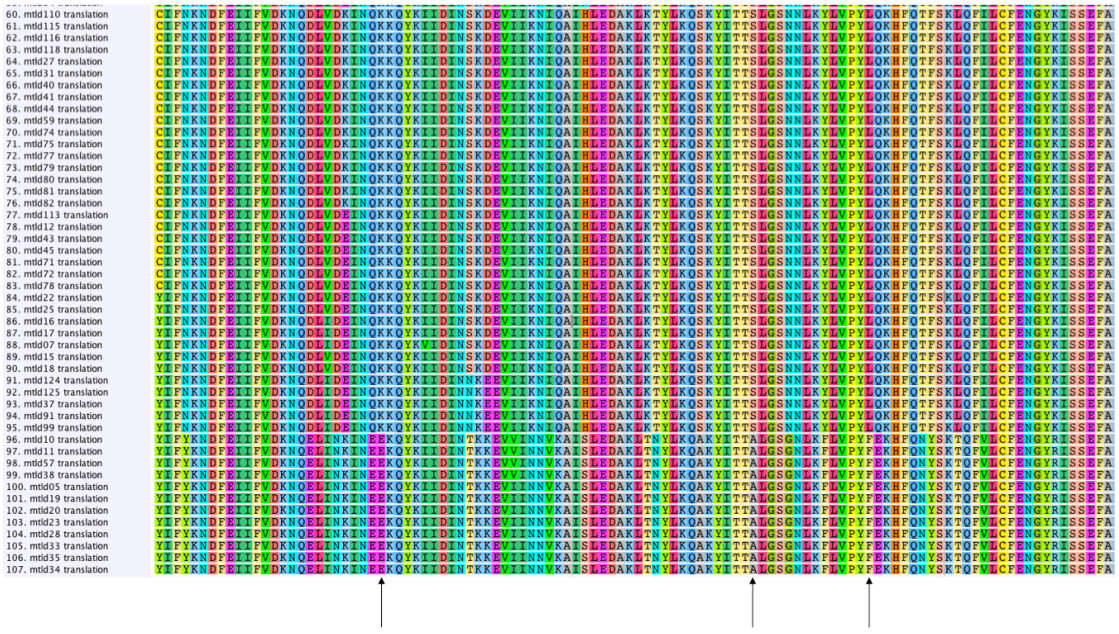
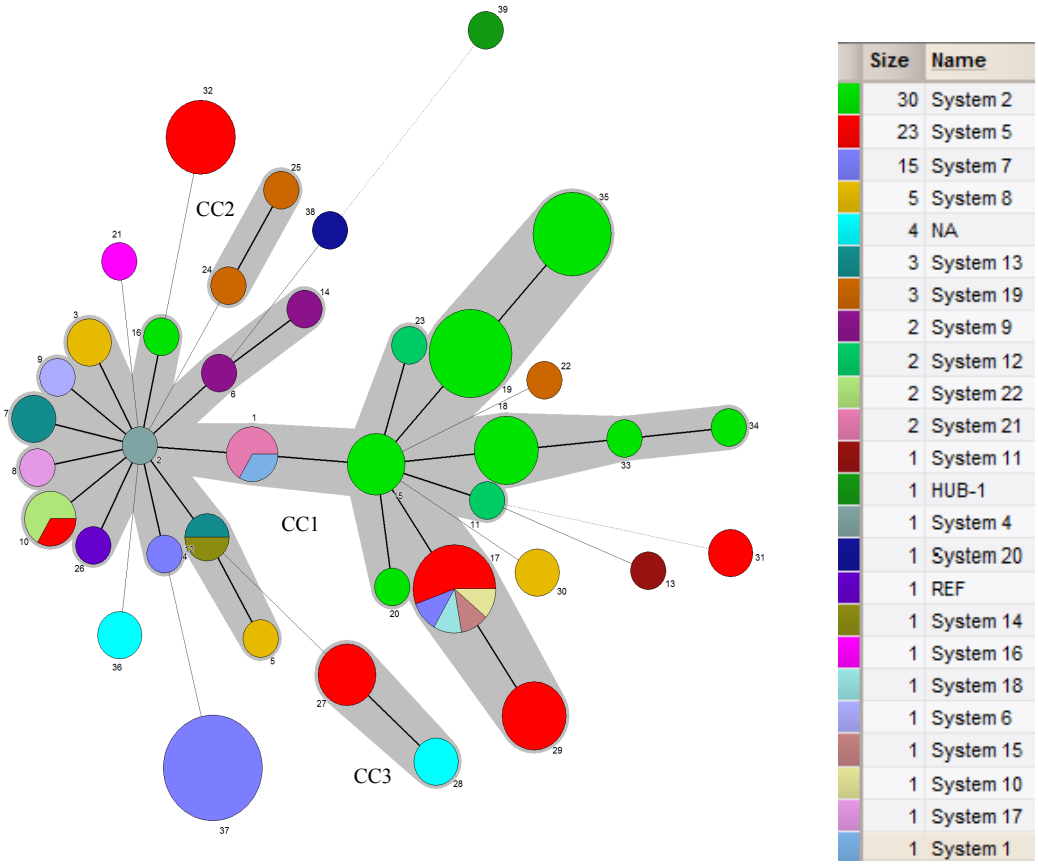
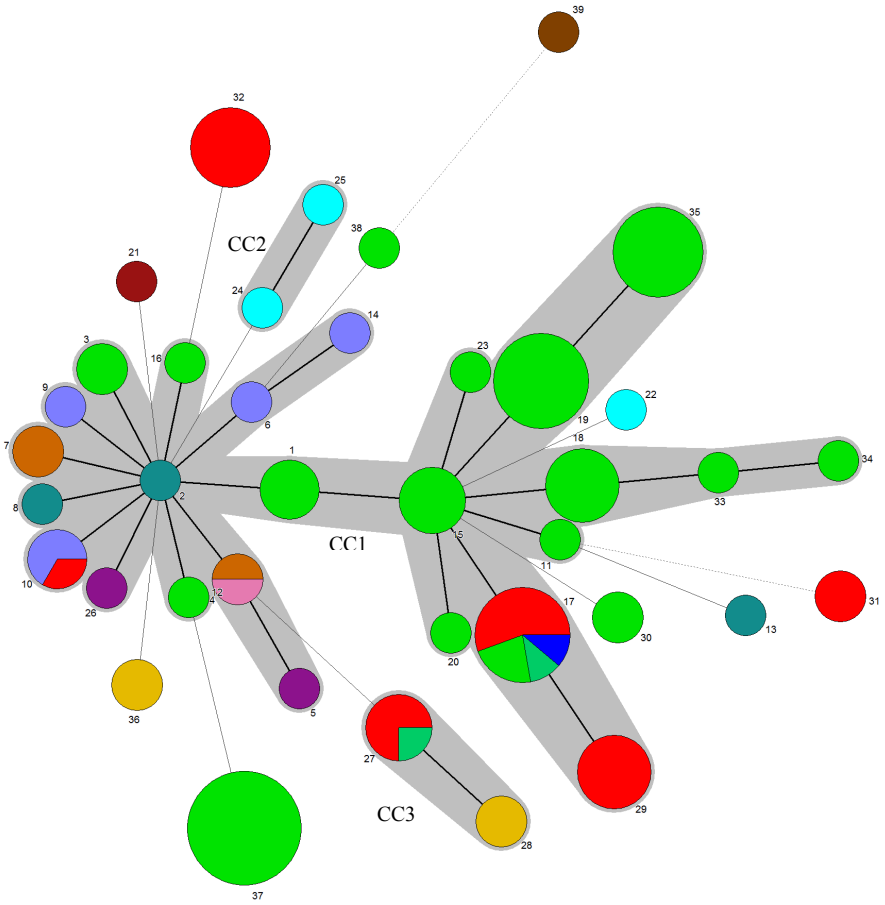


Figure 5.4 Minimum spanning tree analysis for 104 *M. hyorhinis* isolates. Each circle corresponds to a ST. Size of the circle represents numbers of isolates with same ST. Lines between STs indicate inferred phylogenetic relationships. Thickness of the line represents the number of allelic mismatches between the STs (bold=1, plain=2, dotted=3). Grey zones that surround STs belong to the same clonal complex (CC). Number near circles indicates ST number.

(a) Color of the circle indicates the swine production system. Legend: "size" indicates number of isolates and "name" indicates system number.

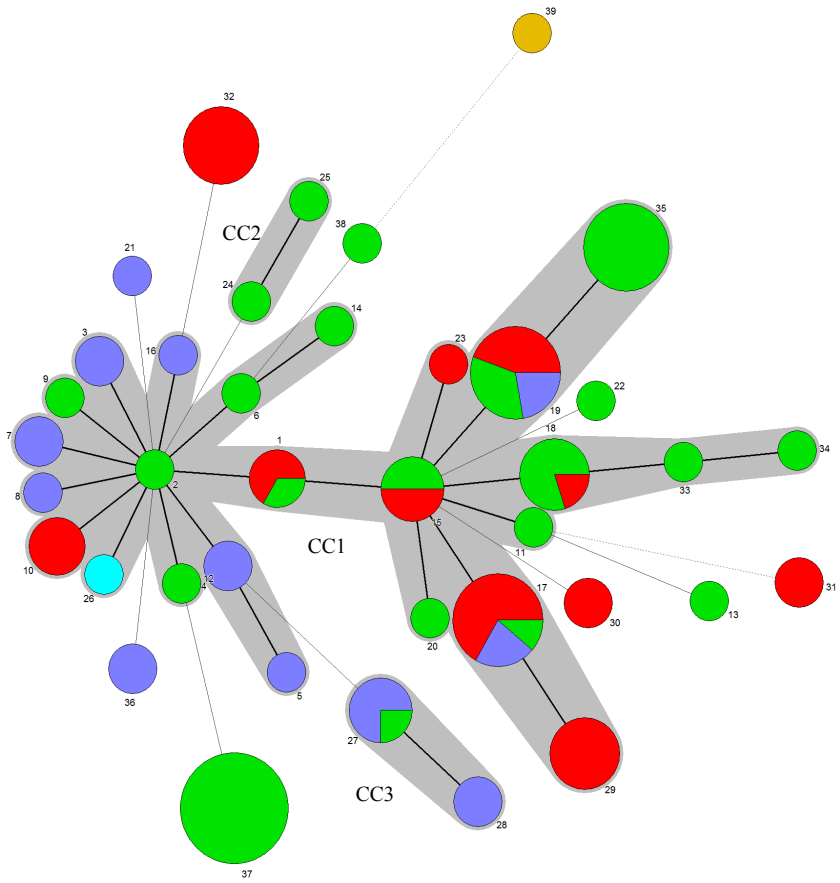


(b) Color of circle represents the geographical location (state)



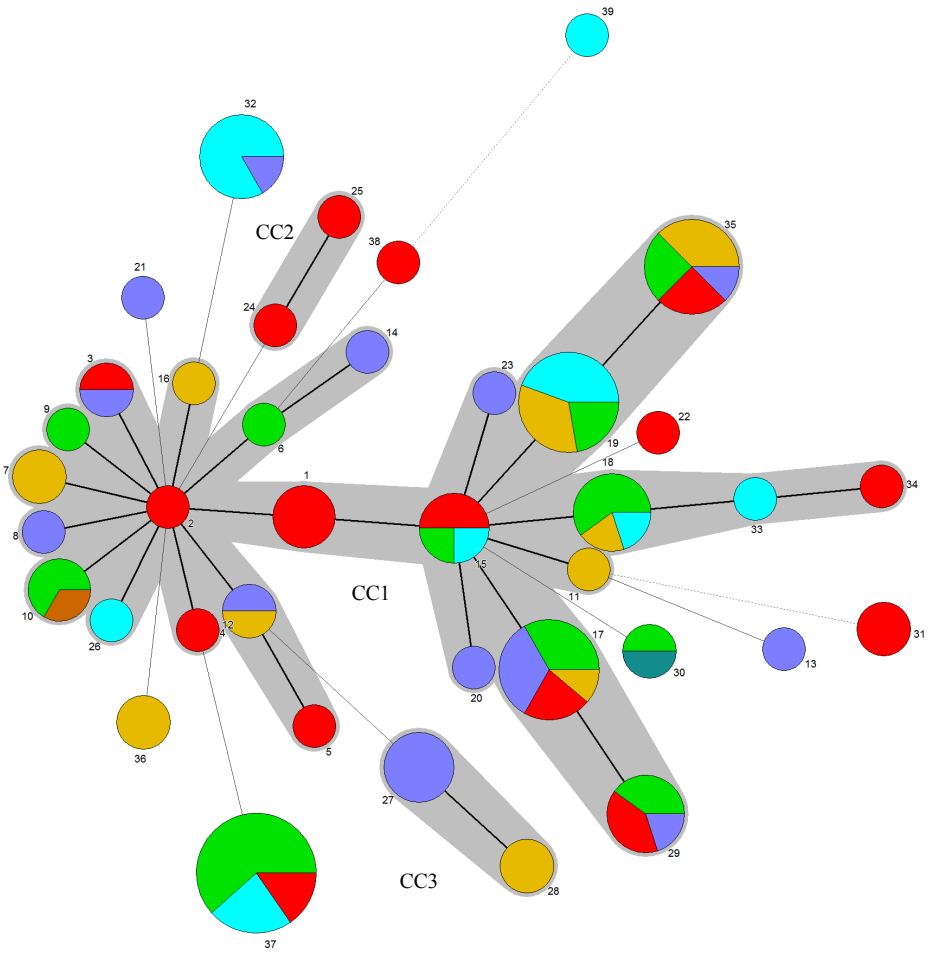
Size	Name
56	MN
22	OK
5	NE
4	NA
3	MEXICO
3	NC
3	IL
2	IA
2	KS
1	CHINA
1	TN
1	PA
1	IN

(c) Color of circle represents the year of isolation (state)



	Size	Name
	47	2011
	34	2012
	21	2010
	1	NA
	1	1973

(d) Color of circle represents type of sample



Size	Name
26	Bronchus
24	Pericardium
18	Pleura
17	Joint
17	Nasal cavity
1	Aerosol
1	Peritoneum

GENERAL DISCUSSION AND CONCLUSIONS

Mycoplasma hyorhinis is a wall-less bacterium commonly attached to the ciliated upper respiratory tract of swine that is able to invade systemic organs causing fibrinous polyserositis and arthritis. More recently, swine veterinarians have raised concerns about the increased involvement of this pathogen in post-weaning morbidity and mortality. The urgent need of information regarding the ecology and epidemiology of the microorganism to support the development of control and prevention programs became evident in 2010 when *M. hyorhinis* was detected by PCR in 55% of polyserositis and 12% of arthritis cases submitted to the Minnesota Veterinary Diagnostic Laboratory. The main goal of this dissertation was to advance the knowledge on the infection dynamics and the epidemiology of *M. hyorhinis* in swine populations in the US.

In Chapter 2, a high-throughput, sensitive and specific quantitative PCR was developed for the detection of *M. hyorhinis* DNA in diverse clinical samples. The assay was shown to be highly sensitive, detecting 12 geq/µl of extracted DNA. High specificity was demonstrated with a large panel of swine bacteria and clinical samples. Furthermore, the repeatability and diagnostic accuracy of the assay was comprehensively evaluated by testing matrices such as oral fluid, nasal swab and serosal surface swab spiked with known amounts of *M. hyorhinis* DNA. Overall, the qPCR showed suitable precision within and between runs. In summary this qPCR represents a valuable tool to characterize infection dynamics and to quantify bacterial load in order to evaluate selected interventions targeting *M. hyorhinis*.

Chapter 3 provided an original opportunity to explore the prevalence of *M. hyorhinis* nasal infection in three large commercial breeding herds and their downstream pig flows. The results consistently showed an extremely low prevalence in the sows and suckling piglets potentially caused by low exposure to the bacterium in the farrowing room and/or by protective effect of colostral immunity in the piglets. The prevalence of nasal infection after weaning increased in two distinct patterns. In two herds a high proportion of PCR-positive pigs were detected shortly after weaning while in the third herd the prevalence of nasal infection remained extremely low until the last week in the nursery when all pigs tested PCR-positive. Different factors such as co-infections, environmental exposure to *M. hyorhinis*, parity-segregation, gilt source, timing of strategic medications and virulence of the *M. hyorhinis* strain could have played a role in the differences observed in infection patterns. Interestingly, young sows were more likely to be PCR-positive, which may indicate a potential role of these sows as a source of infection for suckling piglets. In addition, this study provided the opportunity to confirm the role of *M. hyorhinis* as a cause of systemic disease by performing necropsies in both diseased and healthy pigs. While *M. hyorhinis* was detected in the upper respiratory tract of both healthy and diseased pigs, it was only detected in systemic sites of diseased pigs and frequently associated with the typical lesions of fibrinous serositis.

In spite of the valuable information obtained in Chapter 3, the inherent limitations of a cross-sectional study justified the need to monitor the same pigs overtime in order to

better describe the transmission and spread of *M. hyorhinis*. The longitudinal study described in Chapter 4 confirmed some results of Chapter 3. While the prevalence of *M. hyorhinis* infection observed in suckling piglets and sows was low (<5%), over 90% of the pigs became colonized during the nursery phase. Moreover, the incidence rate estimations demonstrated a relatively high number of pigs acquiring the microorganism within a short period of time, indicating that a rapid transmission occurred after placement in the nursery. In contrast to what was observed before, no significant difference was detected between the proportion of PCR-positive young and old sows in this study, suggesting that a parity effect may be present on some herds only. Interestingly, tonsils from a subset of study pigs were swabbed at slaughter and tested PCR-positive, indicating that a pig infected in the nursery can remain positive until slaughter (~200 days of age).

In Chapter 5, the development of an MLST-s typing scheme for epidemiological and genetic characterization of *M. hyorhinis* field isolates was described. After evaluating more than 20 loci as potential gene targets, the final MLST-s protocol contained 2 housekeeping genes, *pdhB* and *ung,* the surface protein *mtlD* and the adhesin *p95*. This study demonstrated genetic variation within *M. hyorhinis* strains circulating in US swine production systems. The housekeeping genes, *pdhB* and *ung* showed genetic variation of 1.4-2% and 6-7 different allele types, respectively. The surface protein mtlD showed great variation (~ 21%) within the analyzed sequences and more than 20 different alleles.

The MLST-s scheme presented here was shown to be highly discriminatory, capable of subdividing 104 isolates into 39 distinct STs. While no clear relationships were observed between ST and state of origin, sample type and pig age, the majority of isolates from each system clustered together. The majority of STs grouped strains from the same production system, but there were cases where multiple systems shared the same ST, indicating a potential common source of pig flow. On the other hand, multiple STs were found within the same production system and even two different STs were isolated from the same pig. The application of the MLST-s protocol described here will allow studying *M. hyorhinis* epidemiology and dynamics of infection in U.S swine herds. Molecular typing of *M. hyorhinis* will also aid in better understanding transmission routes, in assessing sources of infection and in evaluating interventions such as vaccination and use of antibiotics.

Taken together, the information generated in this thesis provided essential *M. hyorhinis* epidemiological information applicable to modern US swine production systems. Data presented here showed that only a small proportion of swine in sow farms are infected while most of the pigs become infected in the nursery and remain infected through the finishing period. The source of infection for piglets remains unclear, however young sows could play a role in certain herds. There are multiple different strains of *M. hyorhinis* that can be isolated from affected herds and more than one strain can be found in one production system. Forthcoming *M. hyorhinis* research could focus on identifying

risk factors for pathogen transmission and disease manifestation. The tools developed, evaluated and presented here can aid in such studies. Moreover, new information on the prevalence and incidence of infection could be used to better target control measures such as antibiotic and vaccine administration.

REFERENCES

Amass SF, Clark LK, Wu CC. 1995. Source and timing of *Streptococcus suis* infection in neonatal piglets: Implications for early weaning procedures. Swine Health and Prod. 3(5):189–193.

Amass SF, Wu CC, Clark LK. 1996. Evaluation of antibiotics for the elimination of the tonsillar carrier state of *Streptococcus suis*. J Vet Diag Inv 8:64–67.

Assunção P, De la Fe C, Kokotovic B, González O, Poveda J.B. 2005. The occurrence of mycoplasmas in the lungs of swine in Gran Canaria (Spain). Veterinary Research Communications 6: 453-462.

Barker EN, Tasker S, Day MJ, et al. 2009. Development and use of real-time PCR to detect and quantify *Mycoplasma haemocanis* and "Candidatus *Mycoplasma haematoparvum*" in dogs. Microbiol 140:167-170.

Barlev NA, Borchsenius SN. 1991. Continuous distribution of Mycoplasma genome 443 sizes. Biomed. Sci. 2:641-645.

Barthel CH, Duncan JR, and Ross RF. 1972. Histologic and histochemical characterization of synovial membrane from normal and *Mycoplasma hyorhinis* infected swine. Am J Vet Res 33:2501-2510.

Baseman JB, Tully JD. 1997. Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety. Emerg Infect Dis. 1997 Jan-Mar; 3(1): 21–32.

Binder A, van Wees C, Likitdecharote B, Kirchhoff H. 1989. Fluorescence serological and culture detection of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* in swine lungs. Berl Munch Tierarztl Wochenschr 102:11-13.

Bork P, Ouzounis C, Casari G, Schneider R, Sander C, Dolan M, Gilbert M, Gillevet PM. 1995. Exploring the *Mycoplasma capricolum* genome: a minimal cell reveals its physiology. Mol. Microbiol. 16:955–967.

Calcutt MJ, Foecking MF, Fox LK. 2014. Complete Genome Sequence of the Bovine Mastitis Pathogen *Mycoplasma californicum* Strain ST-6T (ATCC 33461T). Genome Announc. 2014 Jul-Aug; 2(4): e00648-14.

Calsamiglia M, Pijoan C. 2000. Colonization state and colostral immunity *Mycoplasma hyopneumoniae* of different parity sows. Vet. Rec. 146, 530–532.

Caron J, Ouardani M, Dea S. 2000. Diagnosis and differentiation of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* infections in pigs by PCR amplification of the p36 and p46 genes. J Clin Microbiol 38:1390-1396.

Carver T, Thomson N, Bleasby A, Berriman M and Parkhill J. 2009. DNAPlotter: circular and linear interactive genome visualization. Bioinformatics 25;1;119-20

Cerdà-Cuéllar M, Naranjo JF, Verge A, Nofrarías M, Cortey M, Olvera A, Segalés J, Aragon V. 2010. Sow vaccination modulates the colonization of piglets by Haemophilus parasuis. Vet Microbiol. 2010 Oct 26;145(3-4):315-20.

Chenna R, Sugawara H, Koike T, et al. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 31:3497-3500.

Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, et al. 2006. Toward automatic reconstruction of a highly resolved tree of life. Science 311: 1283–1287.

Citti C, Nouvel LX, Baranowski E. 2010. Phase and antigenic variation in mycoplasmas. Future Microbiol. 2010 Jul;5(7):1073-85.

Citti C, Watson-McKown R, Droesse M, Wise KS. 2000. Gene families encoding phase- and size-variable surface lipoproteins of *Mycoplasma hyorhinis*. J. Bacteriol. 182,1356–1363.

Citti C, Wise KS. 1995. *Mycoplasma hyorhinis* vlp gene transcription: critical role in phase variation and expression of surface lipoproteins. Mol. Microbiol. 18, 649–660.

Citti C1, Blanchard A. 2013. Mycoplasmas and their host: emerging and re-emerging minimal pathogens. Trends Microbiol 21(4):196-203

Citti, C. Nouvel LX, Baranowski E. 2010. Phase and antigenic variation in mycoplasmas. Future Microbiol. 5(7):1073–1085

Clinical and Laboratory Standards Institute. 2004. Evaluation of precision performance of quantitative measurement methods. Wayne, PA, USA: CLSI; CLSI document EP05-A2.

Cooper J, Feil E. 2004. Multilocus sequence typing – what is resolved? Trnds in Micro. 12(8), 373-377.

Dabrazhynetskaya A, Soika V, Volokhov D, Simonyan V, Chizhikov V. 2014. Genome sequence of Mycoplasma hyorhinis strain DBS 1050. Genome Announc. 2(2):e00127-14. doi:10.1128/genomeA.00127-14.

Darling AE, Mau B, Perna NT. 2010. Multiple Genome Alignment with Gene Gain, Loss, and Rearrangement. PLoS One 5(6), e11147.

Das A, Spackman E, Pantin-Jackwood MJ, Suarez D. 2009. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR. J Vet Diagn Invest 21:771–778.

Duncan JR, Ross RF: 1973, Experimentally induced *Mycoplasma hyorhinis arthritis* of swine: pathologic response to 26th postinoculation week. Am J Vet Res 34:363-366.

Duncan JR, Ross RF. 1969. Fine structure of the synovial membrane in *Mycoplasma hyorhinis* arthritis of swine. Am J Pathol 57:171-186.

Dybvig K, and Voelker LL. 1996. Molecular biology of mycoplasmas. Annu. Rev. Microbiol. 50:25-57.

Falk K, Høie S, Lium B.M. 1991. An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. II. Enzootic pneumonia of pigs: microbiological findings and their relationship to pathomorphology. Acta Vet Scand. 32(1):67-77.

Fano E, Pijoan C, Dee S, Deen J. 2007. Effect of *Mycoplasma hyopneumoniae* colonization at weaning on disease severity in growing pigs. Can. J. Vet. Res. 71, 195–200.

Fraser C, M JD, Gocayne O, White MD, Adams RA, Clayton RD, Fleischmann CJ, Bult AR, Kerlavage G, Sutton JM, Kelley JL, Fritchman JF, Weidman KV, Small M, Sandusky J, Fuhrmann D, Nguyen TR, Utterback DM, Saudek CA, Phillips JM, Merrick JF, Tomb BA, Dougherty KF, Bott PC, Hu TS, Lucier SN, Petterson HO, Smith CA, Hutchison III, Venter JC. 1995. The minimal gene complement of *Mycoplasma genitalium*. Science 270:397–403.

Friis NF, Feenstra AA. 1994. *Mycoplasma hyorhinis* in the etiology of serositis among piglets. Acta Vet Scand 35:93-98.

Friis NF, Kokotovic B, Svensmark B. 2002, *Mycoplasma hyorhinis* isolation from cases of otitis media in piglets. Acta Vet Scand 43:191-193.

Friis NF, Kokotovic B, Svensmark B. 2002. Mycoplasma hyorhinis isolation from cases of otitis media in piglets. Acta Vet Scand. 43(3):191-3.

Friis NF. 1971. Mycoplasmas cultivated from the respiratory tract of Danish pigs. Acta Vet Scand 12:69-79.

Friis, NF. 1976. A serologic variant of *Mycoplasma hyorhinis* recovered from the conjunctiva of swine. Acta Vet Scand. 17(3), 343-353

Gois M, Cerny M, Rozkosny V, Sovadina M. 1968. Production of pneumonia after intranasal inoculation of gnotobiotic piglets with three strains of *Mycoplasma hyorhinis*. J. Comp. Path. 81:401-410

Gois M, Franz J, Kuksa F, et al. 1972. Immune response of gnotobiotic piglets infected with *Mycoplasma hyorhinis*. Zentralbl Veterinarmed B. 19: 379-390.

Gois M, Kuksa F. 1974. Intranasal infection of gnotobiotic piglets with *Mycoplasma hyorhinis*: differences in virulence of the strains and influence of age on the development of infection. Zentralbl Veterinarmed B 21: 352-361.

Gois M, Kuksa F. 1974. Intranasal infection of gnotobiotic piglets with *Mycoplasma hyorhinis*: differences in virulence of the strains and influence of age on the development of infection. Zentralbl Veterinarmed B. 21:352-61.

Gois M, Pospisil Z, Cerny M, Mrva V. 1971. Production of pneumonia afterintranasal inoculation of gnotobiotic piglets with three strains of *Mycoplasma hyorhinis*. J Comp Pathol 81:401-410.

Gois M, Pospisil Z, Cerny M, Mrva V. 1971. Production of pneumonia after intranasal inoculation of gnotobiotic piglets with three strains of *Mycoplasma hyorhinis*. Journal of Comparative Pathology. 81: 401-410.

Gois M, Valícek L, Sovadina M. 1968. *Mycoplasma hyorhinis*, a causative agent of pig pneumonia. Zentralbl Veterinarmed B 15:230-240.

Gois M., Kuksa F. & Sisák F. 1977. Experimental infection of gnotobiotic piglets with Mycoplasma hyorhinis and Bordetella bronchiseptica. Zentralbl Veterinarmed B. 24:89-96.

Gomes Neto JC, Gauger PC, Strait El, et al. 2012. *Mycoplasma* associated-arthritis: critical points for diagnosis. Journal of Swine Health and Production; 20(2):82–86

Goodison S, Urquidi V, Kumar D, Reyes L, Rosser CJ. 2013. Complete genome sequence of Mycoplasma hyorhinis strain SK76. Genome Announc.1(1):e00101-12

Goodwin RF, Pomeroy AP, Whittlestone P. 1965. Production of enzootic pneumonia in pigs with a mycoplasma. Vet. Rec. 77:1247-1249.

Hahn TW, Willby MJ, Krause dc. 1998. HMW1 is required for cytadhesin P1 trafficking to the attachment organelle in *Mycoplasma pneumoniae*. J. Bacteriol. 180:1270–1276.

Haubold H, Hudson RR. 2000. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Bioinformatics 16,847-848.

Hayflick F, Chanock RM. 1965. Mycoplasma species of man. Bacteriol Rev. 29:185-221.

Hayflick L. Tissue cultures and mycoplasmas. 1965. Tex Rept Biol Med 1965; 23(suppl.1):285–303.

Himmelreich R, Hilbert H, Plagens H, Pirkl E, Li BI, Herrmann R.1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. Nucleic Acids Res. 24:4420–4449

Hothorn T, Hornik K, Van de Wiel MA, Zeileis A. 2008. Implementing a Class of Permutation Tests: The coin Package. Journal of Statistical Software 28(8), 1-23. URL http://www.jstatsoft.org/v28/i08/.

Huang S, Li JY, Wu J, Meng L, Shou CC. 2001. Mycoplasma infections and different human carcinomas. World J Gastroenterol 7: 266–269.

Kang I, Kim D, Han K, Seo HW, et al. 2012. Optimized protocol for multiplex nested polymerase chain reaction to detect and differentiate *Haemophilus parasuis*, *Streptococcus suis*, and *Mycoplasma hyorhinis* in formalin-fixed, paraffin-embedded tissues from pigs with polyserositis. Can J Vet Res 76:195–200.

Kawashima K, Yamada S, Kobayashi H, Narita M. 1996. Detection of porcine reproductive and respiratory syndrome virus and *Mycoplasma hyorhinis* antigens in pulmonary lesions of pigs suffering from respiratory distress. J Comp Pathol 114:315-323.

Kawashima K., Katsuda K. & Tsunemitsu H. 2007. Epidemiological investigation of the prevalence and features of postweaning multisystemic wasting syndrome in Japan. J Vet Diagn Invest. 19(1):60-8.

Kenney K, Polson D. 2011. Validation of Swiffer cloth-origin neutralizing broth samples for detection of PRRS virus in the environment. In: Proceedings of the American Association of Swine Veterinarians Conference March 5-8, 2011; Phoenix, AZ: 95-107.

Kinne J, Johannsen U, Neumann R, Mehlhorn G, Pfützner H. 1991. The pathology and pathogenesis of experimental *Mycoplasma hyorhinis* infection of piglets with and without thermomotoric stress. Zentralbl Veterinarmed A. 38(4), 306-20.

Kixmöller M, Ritzmann M, Eddicks M, Saalmüller A, Elbers K. Fachinger V. 2008. Reduction of PMWS-associated clinical signs and co-infections by vaccination against PCV2. *Vaccine.* 26(27-28):3443-51.

Kobayashi H, Morozumi T, Miyamoto C, et al. 1996. *Mycoplasma hyorhinis* infection levels in lungs of piglets with porcine reproductive and respiratory syndrome (PRRS). J Vet Med Sci 58:109-113.

Kobayashi H, Nakajima H, Shimizu Y, Eguchi M, Hata E, Yamamoto K. 2005. Macrolides and lincomycin susceptibility of *Mycoplasma hyorhinis* and variable mutation of domain II and V in 23S ribosomal RNA. J Vet Med Sci. 67(8):795-800.

Kobisch M, Friis NF: 1996, Swine mycoplasmoses. Rev Sci Tech 15:1569-605.

Kornspan JD, et al. 2011. Genome analysis of a *Mycoplasma hyorhinis* strain derived from a primary human melanoma cell line. J. Bacteriol. 193: 4543–4544.

L'Ecuyer C. & Boulanger P. 1970. Enzootic pneumonia in pigs: identification of a causative mycoplasma in infected pigs and in cultures by immunofluorescent staining. Can J Comp Med. 34(1):38-46.

Lau ZJ, Hu BS, Wu WL, Lin ZH, Chang HZ, Shi YZ. 2000. Identification of a Major Cluster of *Klebsiella pneumoniae* Isolates from Patients with Liver Abscess in Taiwan J Clin Microbiol. 38(1), 412–414.

Lefebvre PC, Jones GE, Ojo MO.1987. Pulmonary mycoplasmoses of small ruminants. Rev Sci Tech, Office International DES Epizooties Vol. 6 No. 3 pp. 713-757, 759-799

Leuwerke B. 2009. *Mycoplasma hyorhinis*. Field experiences in diagnosis and control. Proceedings of the Allen Leman Swine Conference. Saint Paul, MN. 36:89-90

Lin JH, Chen SP, Yeh KS, Weng CN. 2006. *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. Vet Microbiol 115:111-116.

Liu W, et al. 2010. Complete genome sequence of *Mycoplasma hyorhinis* strain HUB-1. J. Bacteriol. 192:5844 –5845.

Liu W, Fang, L, Li M, Li S, Guo S, Luo, R, et al. 2012. Comparative genomics of Mycoplasma: analysis of conserved essential genes and diversity of the pan-genome. Plos One, 7(4), e35698.

Lo S. C., M. M. Hayes, H. Kotani, P. F. Pierce, D. J. Wear, P. B. Newton mammalian cells by Mycoplasma penetrans: a newly isolated mycoplasma

Lo SC, Hayes MM, Kotani H, Pierce RF, Wear DJ, Newton PB III, Tully JG, Shih JW. 1993. Adhesion onto and invasion into mammalian cells by *Mycoplasma penetrans*: a newly isolated mycoplasma from patients with AIDS. Mod. Pathol. 6:276–280

Magnusson U, Wilkie B, Mallard B, Rosendal S, Kennedy B. 1998. *Mycoplasma hyorhinis* infection of pigs selectively bred for high and low immune response. Veterinary Immunology and Immunopathology. 61: 83-96.

Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA. 95: 3140-3145.

Maniloff J. 1996. The minimal cell genome: On being the right size. Proc. Natl. Acad. Sci. USA. 93:10004-10006.

Manso-Silván L, Dupuy V, Chu Y, Thiaucourt F. 2011. Multi-locus sequence analysis of *mycoplasma capricolum subsp. capripneumoniae* for the molecular epidemiology of contagious caprine pleuropneumonia. Veterinary Research 2011, 42:86

Manso-Silván L, Dupuy V, Lysnyansky I, Ozdemir U, Thiaucourt F. 2012. Phylogeny and molecular typing of Mycoplasma agalactiae and Mycoplasma bovis by multilocus sequencing. Vet. Microbiol. 2012 Dec 17;161(1-2):104-12.

Mare CJ, Switzer WP. 1965. *Mycoplasma hyopneumoniae*, a causative agent of virus pig pneumonia. Vet. Med. 60:841-846.

Maunsell FP, Woolums AR, Francoz D, Rosenbusch RF, Step DL, Wilson DJ, Janzen ED *Mycoplasma bovis* Infections in Cattle. 2011. ACVIM Consensus Statement. J Vet Intern Med. 25(4):772-83.

Mayor D., J. Jores, B. M. Korczak, and P. Kuhnert. 2008. Multilocus sequence typing (MLST) of *Mycoplasma hyopneumoniae*: A diverse pathogen with limited clonality. Vet. Microbiol. 127:63-72.

McAuliffe L1, Gosney F, Hlusek M, de Garnica ML, Spergser J, Kargl M, Rosengarten R, Ayling RD, Nicholas RA, Ellis RJ. Multilocus sequence typing of *Mycoplasma agalactiae.* J Med Microbiol. 60(Pt 6):803-11

McGill R, Tukey JW, Larsen WA. 1978. Variations of box plots. The American Statistician 32, 12-16.

Meyling A. 1971. Mycoplasma suipneumoniae and *Mycoplasma hyorhinis* demonstrated in pneumonic pig lungs by the fluorescent antibody technique. Acta Vet Scand. 12(1):137-41. Microbiol. Rev. 49:419–455.

Morita T, Muraki Y, Awakura T, Shimada A, Umemura T. 1993. Detection of *Mycoplasma hyorhinis* in porcine eustachitis. J Vet Med Sci. 55(3):475-7.

Morita T., Ohiwa S., Shimada A., Kazama S., Yagihashi T. & Umemura T. 1999. Intranasally inoculated *Mycoplasma hyorhinis* causes eustachitis in pigs. *Vet Pathol.* 36(2):174-8.

Murray D. 2012. Mycoplasma hyorhinis; not just an incidental finding. Proceedings of the Annual Meeting of the American Association of Swine Veterinarians, Denver, Colorado, p 457-459.

Olsen C1, Wang C, Christopher-Hennings J, Doolittle K, Harmon KM, Abate S, Kittawornrat A, Lizano S, Main R, Nelson EA, Otterson T, Panyasing Y, Rademacher C, Rauh R, Shah R, Zimmerman J. 2013 Probability of detecting Porcine reproductive and respiratory syndrome virus infection using pen-based swine oral fluid specimens as a function of within-pen prevalence. J Vet Diagn Invest. 25:328-35

Olvera A, Cerda-Cuellar, Aragon V. 2006. Study of the population structure of Haemophilus parasuis by multilocus sequence typing. Microbiology (2006), 152, 3683–3690

Palzer A, Ritzmann M, Wolf G, Heinritzi K. 2008. Associations between pathogens in healthy pigs and pigs with pneumonia. Veterinary Record. 162: 267-271.

Poland J, Edigton N, Gois M, Betss AO. 1971. The production of pneumonia with or without pleurisy in gnotobiotic piglets with pure cultures of strain TR32 of *Mycoplasma hyorhinis*. Journal of Hygiene 69: 145-154

Potgieter LN, Ross RF. 1972. Demonostration of *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* in lesions of experimentally infected swine by immunofluorescence. Am J Vet Res 33:99-105.

Prickett JR, Kim W, Simer R. 2008. Oral-fluid samples for surveillance of commercial growing pigs for porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 infections. J Swine Health Prod 16:86–91.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Ramirez A, Wang C, Prickett JR, et al. 2012, Efficient surveillance of pig populations using oral fluids. Prev Vet Med 104:292–300.

Razin S, Hayflick L. 2010. Highlights of mycoplasma research: An historical perspective Biologicals. Volume 38, Issue 2, March 2010, Pages 183–190 Special Section: Mycoplasma (pp. 181-248)

Razin S, Yogev D, Naot Y.1998. Molecular Biology and Pathogenicity of Mycoplasmas Microbiology And Molecular Biology Reviews Dec. 1998, p. 1094–1156 Vol. 62, No. 4.

Razin S. 1991. The genera Mycoplasma, Ureaplasma, Acholeplasma, Anaeroplasma, and Asteroleplasma, p. 1937–1959. In A. Balows, H. G.Tru¨per, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, vol. 2, 2nd ed. Springer-Verlag, New York, N.Y.

Razin S. 1992. Mycoplasma taxonomy and ecology, p. 3–22. In J. Maniloff, R. N. McElhaney, L. R. Finch, and J. B. Baseman (ed.), Mycoplasmas: molecular biology and pathogenesis. American Society for Microbiology, Washington, D.C.

Roberts ED, Switzer WP, Ramsey FK. 1963. Pathology of the visceral organs of swine inoculated with *Mycoplasma hyorhinis*. American Journal of Veterinary Research. 24: 9-18.

Romagosa A, Gramer M, Joo HS, Torremorell M. 2011. Sensitivity of oral fluids for detecting influenza A virus in populations of vaccinated and non-vaccinated pigs. Influenza Other Respi Viruses. Mar 2012; 6(2): 110–118.

Rosengarten R, Wise KS. 1990. Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. Science 247, 315–318.

Rosengarten Y, Watson-McKown R, Wise KS. 1991. Molecular basis of Mycoplasma surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5' regulatory sequences. EMBO J. 10(13), 4069-79.

Ross RF, Spear ML. 1973. Role of the sow as a reservoir in infection for *M. hyosynoviae*. Am J Vet Res 34:373-378.

Ross RF, Switzer WP. 1963. Comparison of isolates of *Mycoplasma hyorhinis* by indirect hemagglutination. Am J Vet Res 24:622-627.

Ross RF. 992. Mycoplasmal diseases. In Diseases of swine (A.D. Leman, B. Straw, W. Mengeling, S. D'Allaire & D. Taylor, eds), 7th Ed. Iowa State University Press, Ames, Iowa, 537-551.

Rovira A, Clavijo MJ, Oliveira S. 2010. *Mycoplasma hyorhinis* infection of pigs. Acta Sci Vet 38 (Supl 1):s9-s15.

Ruiz AR, Utrera V, Pijoan C. Effect of *Mycoplasma hyopneumoniae* sow vaccination on piglet colonization at weaning. J Swine Health Prod 2003;11(3):131-135.

Schulman A, Estola T, Garry-Andersson AS. 1970. On the occurrence of *Mycoplasma hyorhinis* in the respiratory organs of pigs, with special reference to enzootic pneumonia. Zentralbl Veterinarmed B 17:549-553.

Shimizu M, Yamada S, Murakami Y, Morozumi T, Kobayashi H, Mitani K, Ito N, Kubo M, Kimura K, Kobayashi M. 1994. Isolation of porcine reproductive and respiratory syndrome (PRRS) virus from Heko-Heko disease of pigs. J Vet Med Sci. 56(2):389-91.

Sibila MA, Nofrarıas M, Lopez-Soria S, Segales J, Riera P, Llopart D, Calsamiglia M. 2007. Exploratory field study on Mycoplasma hyopneumoniae infection in suckling pigs. Veterinary Microbiology 121:352–356

Siqueira FM, Thompson CE, Virginio VG, Gonchoroski T, Reolon L, Almeida LG, Zaha, A. 2013. New insights on the biology of swine respiratory tract mycoplasmas from a comparative genome analysis. BMC Genomics, 14, 175.

Stemke GW, Phan R, Young TF, Ross RF. 1994. Differentiation of *Mycoplasma hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* on the basis of amplification of a 16S 430 rRNA gene sequence. Am J Vet Res 55:81-84.

Sugimura K, Ohno T, Kimura Y, Kimura T, Azuma I. 1992. Arginine deiminase gene of an AIDS-associated mycoplasma, *Mycoplasma incognitus*. Microbiol. Immunol. 36:667–670.

Svenstrup HF, Jørgen SJ, Björnelius E. 2005. Development of a quantitative real-time PCR assay for detection of *Mycoplasma genitalium* J Clin Microbiol 43:3121–3128.

Switzer WP. 1955. Studies on infectious atrophic rhinitis. IV. Characterization of a pleuropneumonia-like organism isolated from the nasal cavities of swine. Am J Vet Res. 16(61 Part 1):540-4.

Tankouo-Sandjong B, Sessitsch A, Liebana E, Kornschober C, Allerberger F, Hächler H, Bodrossy L. 2007. MLST-v, multilocus sequence typing based on virulence genes, for molecular typing of *Salmonella enterica* subsp. enterica serovars. J. Microbiological Methods 69, 23–36.

Taylor-Robinson D. 1975. The importance of mycoplasmas in respiratory infections. Dev Biol Stand. 28:86-100.

Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. Sep; 33(9): 2233–2239.

Ter Laak EA, Pijpers A, Noordergraaf JH, Schoevers EC, Verheijden JH. 1991. Comparison of methods for in vitro testing of susceptibility of porcine Mycoplasma species to antimicrobial agents. *Antimicrob Agents Chemother.* 35(2):228-33.

Thacker EL. 1996. Mycoplasmal Diseases. In: Diseases of Swine. 9.ed. Ames, Iowa: Blackwell Publishing, pp. 701-717.

Timenetsky J, Santos LM, Buzinhani M, Mettifogo E. 2006. Detection of multiple mycoplasma infection in cell cultures by PCR. Braz J Med Biol Res 39:907-914.

Tocqueville VL, Ferré S, Nguyen NH, et al. 2014. MultiLocus Sequence Typing of *Mycoplasma hyorhinis* strains identified by a Real-Time TaqMan PCR assay. J Clin Microbiol (52)5:1664-71.

Truper HG, Dworkin M, Harder W, Schleifer KH. 1992. The prokaryotes, vol. 2, 2nd ed. Springer-Verlag, New York, N.Y.

Van Loo IHM, Heuvelman KJ, King AJ, Mooil FR. 2002. Multilocus Sequence Typing of *Bordetella pertussis* Based on Surface Protein Genes. J Clin Microbiol. 40(6), 1994–2001.

Whitcomb, RF, Tully JG. 1989. The mycoplasmas, vol. V. Spiroplasmas, acholeplasmas, and mycoplasmas of plants and arthropods. Academic Press, Inc., San Diego, Calif.

Williams MV, and Pollack JD. 1990. A mollicute (mycoplasma) DNA repair enzyme: purification and characterization of uracil-DNA glycosylase. J. Bacteriol. 172:2979–2985.

Williams PP. 1978. In vitro susceptibility of *Mycoplasma hyopneumoniae* and *Mycoplasma hyorhinis* to fifty-one antimicrobial agents. Antimicrob Agents Chemother. 14(2):210-3.

Wilson EB. 1927. Probable inference, the law of succession, and statistical inference. Journal of the American Statistical Association 22, pp. 209-212.

Woese CR. 1987. Bacterial evolution. Microbiol. Rev. 51:221–271.

Wu CC, Shryock TR, Lin TL, Faderan M, Veenhuizen MF. 2000. Antimicrobial susceptibility of Mycoplasma hyorhinis. *Vet Microbiol.* 76(1):25-30.

Yang H, Qu L, Ma H, Chen L, Liu W, Liu C, Meng L, Wu J, Shou C. 2010. *Mycoplasma hyorhinis* infection in gastric carcinoma and its effects on the malignant phenotypes of gastric cancer cells. BMC Gastroenterol 10:132.

Yogev D, Rosengarten R., Watson-McKown R. & Wise K.S. 1991. Molecular basis of Mycoplasma surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5' regulatory sequences. EMBO J. 10(13):4069-79.

Yogev D, Watson-McKown R, Rosengarten R, Im J, Wise KS. 1995. Increased structural and combinatorial diversity in an extended family of genes encoding Vlp surface proteins of *Mycoplasma hyorhinis*. J. Bacteriol. 177, 5636–5643

Zadoks, Schukken. 2006. Use of molecular epidemiology in veterinary practice. Vet Clin Food Anim. 22, 229-261.