Mycoplasma hyorhinis and *Mycoplasma hyosynoviae* colonization patterns at various swine production stages

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Luiza Ribeiro Roos

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Dedication

To my parents, Cinara and Julio, my sister, Helena, and my dear fiancé, Ryan.

Abstract

Many microorganisms that are classified as commensal or ubiquitous can cause disease under specific circumstances. Several Mycoplasma species often fall into these categories, including *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae.* Both bacterial species are considered commensal microorganisms of the upper respiratory tract and tonsils of pigs, and are ubiquitous in pig populations, yet capable of causing disease. In nursery age pigs, *M. hyorhinis* causes mainly polyserositis and arthritis, and *M. hyosynoviae* causes arthritis in pigs during the finishing stage. The identification of the origin of colonization, potential animal reservoirs, as well as the timeline of clinical presentation are important aspects in the epidemiology of infectious agents. The present series of studies focus on investigating the association of *M. hyorhinis* and *M. hyosynoviae* colonization status of dams and piglets prior to weaning, and in the case of *M. hyosynoviae* colonization in replacement gilts and in pigs during the wean-to-finish stage. During the lactation period, dams were consistently colonized by both *M. hyorhinis* and *M. hyosynoviae,* although dual colonization did not take place in the majority of sampled dams. Piglets appeared to have a different colonization pattern, however. While *M. hyosynoviae* was hardly detected, *M. hyorhinis* was present in half of piglets sampled at the end of the lactation period. The longitudinal investigation revealed that although *M. hyosynoviae* detection increased with age, the clinical presentation associated with infection decreased in wean-to-finish pigs. In addition, *M. hyosynoviae* colonization was detected in replacement gilts shortly after placement into the gilt development unit. Albeit the increased diagnosis of mycoplasma-associated diseases, the mechanisms of *M. hyorhinis* and *M. hyosynoviae* disease have yet to be unveiled.

Table of Contents

List of Tables

List of Figures

CHAPTER 1
LITERATURE REVIEW

1.1. Introduction

First isolated by Nocard and Roux in 1896 and introduced as genus *Mycoplasma* in 1929 by Nowak, mycoplasmas are small bacteria that lack cell wall (reviewed by Hayflick and Chanock, 1965). Mycoplasmas have a small genome with low G + C content (Bové, 1993), and are part of the class *Mollicutes*, which also accommodates Ureaplasmas, Anaeroplasmas and Acholeplasmas that colonize different animal species, and Spiroplasmas that affect insects, crustaceans and plants (Razin, 1978). *Mollicutes* are recognized as the smallest prokaryotic cells capable of self-replication (Hayflick and Chanock, 1965; Baseman and Tully, 1997), measuring between 0.2 and 0.8μm (Hayflick and Chanock, 1965; Freundt, 1983).

Members of this class include several mycoplasmas that can be detected in various host species, and those that are specifically isolated from swine, such as *Mycoplasma hyorhinis* (*M. hyorhinis*; Switzer, 1955), *M. hyopneumoniae* (Mare and Switzer, 1965; Goodwin et al., 1965), *M. hyosynoviae* (Ross and Karmon, 1970), and *M. flocculare* (Meyling and Friis, 1972). Other mycoplasmas that have been reported as colonizing or causing disease in pigs are *M. suis* (former *Eperythrozoon suis*; Splitter, 1950; Neimark et al., 2002), *M. granularum* (Switzer, 1964), *M. hyogenitalium* (Moore et al., 1966), *M. hyoarthrinosa* (Robinson et al., 1967), *M. laidlawii* (Taylor-Robinson and Dinter, 1968), and *M. hyopharyngis* (Erickson et al., 1986).

Among the mycoplasmas isolated from swine, the ones recognized as being more significant in the modern day swine industry, and capable of causing disease include *M. hyopneumoniae*, *M. hyosynoviae,* and *M. hyorhinis* (reviewed by Kobisch and Friis,

1996). *Mycoplasma hyorhinis* mainly causes polyserositis and arthritis in young pigs (Roberts et al., 1963), *M. hyopneumoniae* has been established as the primary cause of enzootic pneumonia in growing pigs (Mare and Switzer, 1965; Goodwin et al., 1965), and *M. hyosynoviae* was identified as a cause of arthritis in growing pigs (Ross and Karmon, 1970).

1.2. *Mycoplasma hyorhinis* infection in swine

1.2.1. Etiology

Described by Switzer (1955), *M. hyorhinis* resides in the class *Mollicutes*, microorganisms that are characterized by the absence of cell wall due to genetic incapability to synthetize peptidoglycan (reviewed by Waites and Talkington, 2004), which allows it to be highly pleomorphic (Hayflick and Chanock, 1965). *Mycoplasma hyorhinis* is the first swine mycoplasma that grows in culture and easily becomes predominant in the process of swine mycoplasmal disease investigation (reviewed by Thacker, 2009). Like other mycoplasmas, *M. hyorhinis* requires rich media for successful growth (Hayflick and Chanock, 1965), usually composed by blood serum and yeast extract. Once plated in solid agar, the formed colonies display the characteristic "fried-egg" morphology after two to five days of growth (Kobisch and Friis, 1996). The ultrastructure of *M. hyorhinis* has been described *in vitro* (Baskerville et al., 1972) and *in vivo* (Baskerville and Wright, 1973) through electron microscopy, in addition to the identification of two replication methods, binary fission and elementary bodies formation.

In the last years, several *M. hyorhinis* strains have been sequenced, isolated from the respiratory tract (Liu et al., 2011), and joints of pigs (Goodison et al., 2013), and from cell cultures (Kornspan et al., 2011; Calcutt et al., 2012; Dabrazhynetskaya et al., 2014). However, potential differences in pathogenicity and virulence among isolates have been long suggested (Gois and Kuksa, 1974; Whittlestone, 1979), and heterogeneity within *M. hyorhinis* isolates has been proposed with the use of pulsed-field gel electrophoresis (PFGE; Barlev and Borchsenius, 1991; Yamaguti et al., 2015). Antigenic differences between distinct *M. hyorhinis* isolates were first demonstrated by seroreactivity to specific antisera (Friis, 1971), and later by growth-inhibition, metabolism-inhibition, latex-agglutination and polyacrylamide-gel-electrophoresis tests (Gois et al., 1974). Additionally, a few surface antigens of *M. hyorhinis* have been identified densely distributed on the surface of the microorganism by the use of surface biding monoclonal antibodies (Wise and Watson, 1983a; Wise and Watson, 1983b; Boyer and Wise, 1989), with some reported to be strain specific (Wise and Watson, 1983a). More recently, *M. hyorhinis* genetic variation and recombination has been demonstrated by multilocus sequence typing (MLST; Tocqueville et al., 2014) and multiple-locus variable-number tandem-repeat analysis (MLVA; Dos Santos et al., 2015).

Mycoplasma hyorhinis posses an elaborate family of multiple genes that encode the membrane lipoproteins, identified as the variable surface lipoprotein (Vlp) system (Rosengarten and Wise, 1990; Yogev et al., 1991). Each Vlp gene is described containing characteristic open reading frames that span the coding sequence of the mature surface-exposed lipoproteins, which provide a potential reservoir of coding sequence for Vlp

diversity, possibly recruited through insertion and/or deletion mutations (Yogev et al., 1995; Citti et al., 2000). *In vitro* Vlps undergo spontaneous high frequency random mutations, both in repetitive structural domains and in their 5' promoter regions, that determine phase variation in expression and size variation (Rosengarten and Wise, 1990; Rosengarten and Wise, 1991; Yogev et al., 1991; Citti et al., 2000). Size variation of Vlp products results from in-frame insertion or deletion of tandemly repeated intragenic sequences within the 39 regions of individual Vlp genes, thereby contracting or expanding the surface-exposed C-terminal region of the corresponding Vlp (Rosengarten and Wise, 1991; Yogev et al., 1991). A distinct mutational event is associated with high frequency phase variation in the expression of individual Vlp products, operating independently for each Vlp (Citti et al., 2000). This feature allows individual microorganisms to co-express multiple Vlps, resulting in distinct Vlp surface mosaics (Rosengarten and Wise, 1991).

Thus far, the products VlpA, VlpB, VlpC, VplD, VplE and VplF have been documented (Rosegarten and Wise, 1990; Rosengarten et al., 1993; Yogev et al., 1995) yet, the full extent of the Vlp gene repertoire is still under investigation. These constitute the major membrane protein and surface coat of the organism, which is the primary interface with the external environment (Yogev et al., 1991), and is suggested as being the main component for antigenic and structure variation in *M. hyorhinis,* as well as immune system evasion (Rosengarten and Wise, 1991). It has been proposed that the surface lipoprotein region more proximal to the membrane contains epitopes recognized by surface-binding antibodies (Cleavinger et al., 1994), in addition to reports of the

effectiveness of Vlp size variation in conferring resistance to host antibodies and identifying mutational pathways within the Vlp system that can be mobilized to maintain protective Vlp profiles in populations propagating *in vitro* (Cleavinger et al., 1994; Citti et al., 1997). More recently, the cytoadhesion ability of the Vlp system was demonstrated *in vitro* by direct adhesion assay (Xiong et al., 2016).

Mycoplasma hyorhinis is also identified as a cell culture contaminant (Robinson and Wichelhausen, 1956; Drexler and Uphoff, 2002). It has been reported that about 60% of *M. hyorhinis* strains detected as contaminant in cell lines are characterized as cultivar α, that not usually grows in typical cell-free media used in mycoplasma isolation assays (DelGiudice et al., 1980). A strain of cultivar α has been long isolated and identified in Vero cells (Hopps et al., 1973), and, more recently, sequenced (Dabrazhynetskaya et al., 2014). The detection of cell culture contamination might be difficult since, even in high concentration, *M. hyorhinis* does not usually cause cytopathic effect or turbidity (Thornton, 1986), although severe cytopathic effect was observed in swine synovial (Potgieter et al., 1972) and swine testicle cell culture (Shin et al., 2003). However, multiplex polymerase chain reaction (PCR) assays developed for detection of swine Mycoplasmas have dramatically increased sensitivity and detection rate of *M. hyorhinis* in cell cultures (Timenetsky et al., 2006).

1.2.2. Pathogenesis and clinical presentation

Mycoplasma hyorhinis is considered a ubiquitous microorganism in swine production systems, and predominantly colonizes the upper respiratory tract and tonsils

of pigs (Gois ans Kuksa, 1974; Friis and Feenstra, 1994), by adhering to ciliated epithelial cells surface, similarly to *M. hyopneumoniae* and other mycoplasmas (reviewed by Razin and Jacobs, 1992). The mechanisms of systemic spread are unknown, however. *M. hyorhinis* can be isolated from the conjunctiva (Friis, 1976) and blood (Gois et al., 1977) of infected pigs. In young pigs of less than 8 weeks of age, *M. hyorhinis* can cause polyserositis and infectious arthritis (Roberts et al., 1963; Gois et al., 1977). *Mycoplasma hyorhinis* is also suggested as being a causative agent of eustachitis (Morita et al., 1993; Morita et al., 1999), otitis media (Kazama et al., 1994; Morita et al., 1995; Morita et al., 1998), abortions (Shin et al., 2003), and pneumonia in pigs (Schulman and Estola, 1966; Gois et al., 1967; Lin et al., 2006), although this later aspect is contentious (Mare and Switzer, 1965).

Mycoplasma hyorhinis-induced polyserositis lesions are characterized by fibrinous pericarditis, with adhesion formation, pleuritis and peritonitis (Roberts et al., 1963; Gois et al., 1977). It has been reported that acute arthritis is seen in the first two months after *M. hyorhinis* experimental inoculation (Barden and Decker, 1971), with severe fibrinopurulent polyserositis observed in the first 3 weeks (Roberts et al., 1963; Ennis et al., 1971). At microscopic analysis, macrophages and lymphocytes are observed infiltrating the pleura and peritoneal surface, with neutrophil concentration in the areas of attachment (Roberts et al., 1963).

In addition, it has been suggested that *M. hyorhinis* can persist in joints for 2 months (Roberts et al., 1963), although others describe a more chronic persistence, with the pathogen being recovered from joints for 8 months after inoculation (Barden and

Decker, 1971). Three months after inoculation, synovial membrane hypertrophy and pannus formation might be present (Ennis et al., 1971). Microscopic lesions observed in *M. hyorhinis*-induced arthritis suggest a mononuclear cell infiltration in the synovial lining (Duncan and Ross, 1969; Ennis et al., 1971). Neutrophils seem to be the predominant cell type in the synovial fluid, and fibrinous exudation and accumulations of erythrocytes and platelets suggest an inflammatory nature of the early lesion (Duncan and Ross, 1969). In experimental studies it was observed an alteration in the protein content of synovial fluid of pigs with *M. hyorhinis* induced arthritis (Barden and Decker, 1971; Barthel et al., 1972), with decreased albumin and increased γ-globulin content in serum and synovial fluid. Synovial fluid protein titers exceeded those of serum, suggesting local antibody production as a response to infection with immunoglobulin production (Barden and Decker, 1971; Barthel et al., 1972).

Mycoplasmas are described as a mild antigen, although capable of eliciting an immune response by the host (Gois, 1968). Serum of pigs experimentally and naturally infected with *M. hyorhin*is is reported to show significant increase in metabolic-inhibiting antibodies (Gois, 1968; Gois et al., 1969; Ross et al., 1973), first detected at six weeks post infection, with peak titers observed at twelve weeks post infection and decreasing by twenty-six weeks post infection (Ross et al., 1973). When evaluated by complement fixation, antibodies appeared to be detected in serum within two weeks post infection, with peak titers at eight weeks post infection, also decreasing by twenty-six weeks post infection (Ross et al., 1973). Ross et al. (1973) reported a higher antibody concentration detected in synovial fluid in comparison with serum, and also detection of moderate

concentration of metabolic-inhibiting and complement-fixing antibodies still after a year post infection. Evaluated by ELISA, initial seroconversion is observed at around fifteen days post experimental inoculation (Gomes Neto et al., 2014), same period observed by latex agglutination test (Gois et al., 1972). Immunoglobulin G is observed as the main component of the humoral immune response to *M. hyorhinis* infection (Gois et al., 1972; Ross et al., 1973). The surface lipoproteins and lipopeptides of *M. hyorhinis,* likely derived from VlpC, have been suggested as the main causative agent of inflammatory reactions as they stimulate cytokine production by macrophages (Muhlradt et al., 1998).

Clinical signs may not be seen in all affected pigs (Ross, 1992), as, under field conditions, the systemic spread and disease manifestation might be associated with multiple factors (Friis and Feenstra, 1994). Although *M. hyorhinis* can be re-isolated from joints in experimental studies, it might not be present in synovial tissue (Duncan and Ross, 1969). In addition, synovial fluid protein titers might exceed those of serum even in the absence of viable microorganisms in joints (Barden and Decker, 1971; Ennis et al., 1971).

Mycoplasma hyorhinis co-infections have been previously investigated. Pigs infected with *Bordetella bronchiseptica* and *M. hyorhinis* seem to develop more severe signs of disease, with severe polyserositis, polyarthritis and increased mortality, than the ones infected with one of the two pathogens only (Gois et al., 1977). Co-infections of *M. hyorhinis* and porcine reproductive respiratory syndrome virus (PRRSv) are reported as producing more severe pneumonia than PRRSv alone (Shimizu et al., 1994; Kawashima et al., 1996; Kobayashi et al., 1996a). Finally, in co-infections of *M. hyorhinis* and

porcine circovirus type 2 (PCV2) more severe respiratory clinical signs and lung lesions might be observed (Kawashima et al., 2007; Chen et al., 2016). All mentioned studies suggest that *M. hyorhinis* might play a role as a risk factor or potentiating infections by other pathogens.

1.2.3. Transmission dynamics

Mycoplasma hyorhinis is assumed be transmitted from dam to piglets shortly after birth and between weaned pigs by direct contact (reviewed by Friis and Feentra, 1994; Kobisch and Friis, 1996). Polyserositis due to *M. hyorhinis* infection is mainly observed in post-weaned pigs until 8-10 weeks of age, 3 to 10 days after exposure (reviewed by Kobisch and Friis, 1996). In older pigs, between 3 to 6 months of age, *M. hyorhinis* infection usually results in arthritis only (Potgieter and Ross, 1972), and susceptibility to infection seems to decrease with age (Gois and Kuksa, 1974). Genetic susceptibility has also been suggested when miniature pigs experimentally inoculated with *M. hyorhinis* developed a limited and mild acute arthritis (Barden et al., 1973). However, information describing transmission dynamics and epidemiology of *M. hyorhinis* under field conditions is scarce.

1.2.4. Diagnostic samples and association with clinical disease

Mycoplasma hyorhinis diagnosis in field conditions is based on the observation of clinical signs and macroscopic lesions, still other microorganisms can produce the same

type of fibrinous lesions and arthritis, such as *M. hyosynoviae*, *Haemophilus parasuis* or *Streptococcus suis* (reviewed by Thacker, 2009). Microscopic lesions observed in affected tissue are characterized by mono and polymorphonucelar cells in serosal and synovial membrane (Roberts et al., 1963; Duncan and Ross, 1969; Ennis et al., 1971). Differentiation from other bacterial pathogens and isolation can be achieved by culture in specific media (Hayflick and Chanock, 1965) and various samples have been used for this purpose, such as oral fluids, nasal fluids, lung samples, tonsil tissue, and synovial fluids (Gois et al., 1969; Friis and Feesntra, 1994; Makhanon et al., 2012).

Serological tests used in research, specifically, have been described (Roberts et al., 1963; 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, there are no standard serological techniques or tests commercially available for *M. hyorhinis* specific antibody detection, although a patent has been filed with the World Intellectual Property Organization by Lawrence et al. (2016) for a enzyme-linked immunosorbent assay (ELISA), and attempts have been made to adapt commercial ELISA tests used for *M. hyopneumoniae* antibody detection, with seroconversion observed 15 days post *M. hyorhinis* nasal challenge (Gomes Neto et al., 2014). Other tests that have been explored for *M. hyorhinis* detection include immunofluorescence assays (L'Ecuyer and Boulanger, 1970; Gois et al., 1971; Potgieter and Ross, 1972; Jansson, 1974), with reports of lower sensitivity than culture (Binder et al., 1989), and more recently, *in situ* hybridization in formalin-fixed tissue (Boye et al., 2001; Kim et al., 2010).

Several molecular techniques have been developed in the effort to detect and

identify *M. hyorhinis*, including DNA hybridization (Taylor et al., 1984, Taylor et al., 1985), traditional, multiplex, and real time PCR assays (Stemke et al., 1994; Caron et al., 2000; Lin et al., 2006; Stakenborg et al., 2006; Timenetsky et al., 2006; Kang et al., 2012; Clavijo et al., 2014), MLST (Tocqueville et al., 2014) and MLVA (Dos Santos et al., 2015). However, the majority of these assays have been applied in research settings, limiting their diagnostic applicability. It has been proposed that molecular detection can be achieved with various sample types, such as nasal swabs, tonsil scrapings, lung samples, pericardium, synovial fluids and broth culture (Lin et al., 2006; Makhanon et al., 2012; Clavijo et al., 2014; Gomes Neto et al., 2014; Gomes Neto et al., 2015). Gomes Neto et al. (2014) specifically reports the observation of positive nasal swab samples 18 days post *M. hyorhinis* nasal challenge.

Disease caused by *M. hyorhinis* can be difficult to diagnose in the nursery stage since this microorganism is considered a commensal of the upper respiratory tract of pigs (Gois ans Kuksa, 1974; Friis and Feenstra, 1996), and isolation of *M. hyorhinis* from diseased animals does not correspond undoubtedly with the infectious process observed (Rovira et al., 2010; Gomes Neto et al., 2012). In addition, *M. hyorhinis* might not be detected in all arthritic joints of older pigs, with a study reporting the isolation of the microorganism in only 7% of 67 arthritic joints in slaughter pigs in Canada (Hariharan et al., 1992). A combination of herd history, gross and microscopic lesions, and identification of the pathogen seem to be necessary for *M. hyorhinis* infection diagnosis.

1.2.5. Control strategies

Mycoplasma hyorhinis infection is difficult to control and eliminate at the farm level, since the microorganism is ubiquitous of the swine respiratory tract (Gois and Kuksa, 1974), and infection is likely transmitted to piglets by dams prior to weaning (reviewed by Kobisch and Friis, 1996). No commercial vaccines are available for *M. hyorhinis* control, although a patent has been filed in the United States by Lin and Weng (2005) for a Mycoplasma vaccine, and various commercial laboratories offer the option of autogenous vaccine preparation.

Mycoplasmas are naturally resistant to β-lactams, such as penicillin and cephalosporin, and vancomycin due the absence of cell wall, although, *M. hyorhinis in vitro* susceptibility to antimicrobial agents has been previously reported (Ogata et al., 1971; Williams, 1978; Aarestrup et al., 1998; Wu et al., 2000). These authors suggested *M. hyorhinis* being mostly susceptible to macrolides, lincomycin, and tetracycline, and resistant to erythromycin. Yet, one study reported various *M. hyorhinis* strains with decreased susceptibility to macrolides in Japan (Kobayashi et al., 1996b), and it has been suggested that the overuse of these antimicrobials might be involved with the resistance process to macrolides observed over the years (Kobayashi et al., 2005). The efficiency of antimicrobials under field conditions and in currently circulating strains has yet to be investigated *in vivo*, however, *in vitro* susceptibility of *M. hyorhinis* field isolates recovered from cultured lungs has been evaluated in Korea, where the microorganism was observed to be susceptible to lincomycin and resistant to erythromycin (Jin et al., 2013), in agreement with previous studies.

Certain strains of mycoplasma have been shown to be capable of cytoplasmatic invasion within cell membrane vesicles (Hayflick and Stinebrig, 1960), and it has been suggested that *M. hyorhinis* is capable of invading cultured melanoma cells *in vitro* and be found within the cell structure, possibly evading antimicrobial effects (Kornspan et al., 2010). Nonetheless, the *M. hyorhinis* cell invasion capability has yet to be evaluated within the pathogenicity aspect in pigs.

Mycoplasma hyorhinis is worldly distributed and omnipresent in the swine industry and, in addition to the nature of the microorganism as commensal in the upper respiratory tract of pigs (Gois and Kuksa, 1974), not all systems will find necessary the investment of treatment and control. The cost effectiveness of antimicrobial treatments for *M. hyorhinis* control should be a tailored decision made by practitioners and swine producers for specific farms.

1.3. *Mycoplasma hyosynoviae* infection in swine

1.3.1. Etiology

Mycoplasma hyosynoviae is a host-specific bacterium that affects swine, and was named "synovia" due to the location where it was first isolated in pig, in the joints (Ross and Karmon, 1970). Similarly to *M. hyorhinis*, *M. hyosynoviae* belongs to the class *Mollicutes* and was differentiated biologically, serologically and electrophorectically from *Acholeplasma granularum* (former *M. granularum*) by Ross and Karmon (1970). These authors identified *M. hyosynoviae* as α-hemolytic and arginine utilizing-sterol requiring microorganism. This microorganism did not seem to arise from the same

lineage of other swine mycoplasmas, such as *M. hyopneumoniae* and *M. hyorhinis*, instead *M. hyosynoviae* is a member of the *Mycoplasma hominis* group and closely related to *M. orale*, a human commensal (Blank et al., 1996).

Field isolates of different *M. hyosynoviae* strains have been shown to be genomically heterogeneous and not clustered by amplified fragment length polymorphism analysis and pulsed-field gel electrophoresis, even when isolates are collected in the same site (Kokotovic et al., 2002a; Kokotovic et al., 2002b). More recently, the genome sequence of seven different strains of *M. hyosynoviae* has been reported, and certain virulence factors have been identified (Bumgardner et al., 2015). Those virulence factors are related to the Opp proteins, with functions of ATP and oligopeptide transportation, and adhesion mechanisms.

Different isolates of *M. hyosynoviae* have been proposed to have distinct levels of virulence (Gomes Neto et al., 2016), and that would reflect in its capability of becoming systemic and producing clinical disease (Ross et al., 1971), although various strains might cause persistent infection (Ross and Spear, 1973).

1.3.2. Pathogenesis and clinical presentation

Mycoplasma hyosynoviae is considered a commensal of the oral cavity and nasal passages of pigs (Ross and Spear, 1973; Friis et al., 1991), although *M. hyosynoviae* has pathogenic capabilities, as recognized by Ross and Duncan (1970), as arthritis inducer in 3 to 6 months old pigs. *Mycoplasma hyosynoviae* might also contribute as a secondary pathogen to pre existing pneumonia development, and it can be cultivated from the

affected lungs (Friis, 1971; Assunção et al., 2005). Various studies describe *M. hyosynoviae* colonizing the respiratory tract of pigs and, more frequently, chronically persisting in tonsils (Ross et al., 1971; Ross and Spear, 1973; Friss et al., 1991; Hagedorn-Olsen et al., 1999b) and pharynx (Ross and Spear, 1973). However, the determinant factors for tonsillar and pharynx tropism, systemic spread, and disease development have yet to be described. *Mycoplasma hyosynoviae* appears to reach the synovial membranes haematogenously from tonsils and nasal mucosa (Kobisch and Friis, 1996; Hagedorn-Olsen et al., 1999c; Lauritsen et al., 2008) and joint infection can be preceded by bacteremia (Hagedorn-Olsen et al., 1999c).

Pathological findings in *M. hyosynoviae* arthritis are suggestive of lesions confined to the synovial membrane (Hagedorn-Olsen et al., 1999a), characterized by a non-purulent serofibrinous polyarthritis generally observed in growing and finishing pigs that persists acutely for 3 to 10 days decreasing gradually (Hagedorn-Olsen et al., 1999a; Nielsen et al., 2001; Thacker, 2009). *Mycoplasma hyosynoviae* lesions have been previously described by Hagedorn-Olsen et al., (1999a) and Nielsen et al., (2001). During the acute phase of infection, between 6 to 15 days after exposure, synovial membranes are affected by moderate to severe proliferation and edema with yellowish or reddish-brown turbid synovial fluid. Changes in coloration and turbidity of synovial fluid might also be present in the chronic phase, as well as synovial membrane proliferation.

The consequent clinical presentation of the arthritis infection process is observed as lameness (Ross and Duncan, 1970; Ross et al., 1971; Nielsen et al., 2001) that might be associated with pain, compromising the welfare of pigs (Nielsen et al., 2001).

However, clinically affected pigs can recover spontaneously (Hagedorn-Olsen et al., 1999b; Nielsen et al., 2001), with no further consequences or mild stiffness of motion (reviewed by Thacker, 2009). It has also been proposed that *M. hyosynoviae* can be present in joints grossly normal at necropsy (Ross, 1972) and with no apparent clinical presentation (Nielsen et al., 2001; Nielsen et al., 2005). In addition, osteochondrosis has been suggested to be a predisposing factor for *M. hyosynoviae* establishment and infection in the joint (Lawrisuk et al., 1987), although this hypothesis has not been supported by more recent studies (Nielsen et al., 2001).

1.3.3. Transmission dynamics

Mycoplasma hyosynoviae seems to be introduced to naïve herds by chronically infected carrier replacement gilts (Ross and Spear, 1973), even though information about *M. hyosynoviae* epidemiology is sparse. The main *M. hyosynoviae* transmission route is proposed to be the direct contact between colonized adult pigs and susceptible pigs during the acute phase of infection (Ross and Spear, 1973). However, more recently, other authors have proposed that *M. hyosynoviae* can spread throughout the herd from acute or sub acute as well as chronically infected pigs (Hagedorn-Olsen et al., 1999b; Hagedorn-Olsen et al., 1999c).

It has been suggested that *M. hyosynoviae* is not consistently capable of being transmitted to pigs younger than 4-8 weeks of age (Ross and Spear, 1973). Yet, no evidence of age-related restriction in susceptibility to *M. hyosynoviae* arthritis was found in experimentally infected 6-week old naïve pigs (Lauritsen et al., 2008). Also, recent

studies have suggested that tonsillar colonization of suckling pigs might occur prior to weaning (Hagedorn-Olsen et al., 1999c; Schwartz et al., 2014), in contrast to what has been previously reported (Ross and Duncan, 1970).

Maternal derived antibodies against *M. hyosynoviae* may play a role in the protection of disease development in piglets prior to weaning (Ross and Spear, 1973), since a small portion of piglets act as carriers post weaning and are capable of transmitting the pathogen to pen mates during growing and finishing stages (Hagedorn-Olsen et al., 1999c). The colonization pattern post weaning seems to be inconsistent and vary greatly among individual pigs, with some animals becoming carriers before 8 weeks of age and most others not before 18 weeks of age (Hagedorn-Olsen et al., 1999c). Disease mortality is described as being usually low, although morbidity may reach 50% in affected herds (Ross, 1992), however, higher mortality rates might be associated with the euthanasia of animals with severe lameness and compromised welfare.

1.3.4. Diagnostic samples and association with clinical disease

Mycoplasma hyosynoviae diagnosis might be achieved by several different approaches, although there is not an established standard method. Culture and isolation have been used since *M. hyosynoviae* was first isolated (Ross and Karmon, 1970) and can be laborious as for other mycoplasmas (Friis et al., 1991). The culture media is based on the same components used in media for culture of other swine mycoplasmas, such as blood serum and yeast extract (Hagedorn-Olsen et al., 1999a), with the addition of arginine or mucin (Friis et al., 1991). *Mycoplasma hyosynoviae* can be isolated from

synovial fluids and tonsils of colonized pigs (Hagedorn-Olsen et al., 1999a; Nielsen et al., 2001; Makhanon et al., 2012).

Nielsen et al. (2001) have reported a *M. hyosynoviae* isolation rate of 20% from joints of lame pigs, similarly to previous studies (Friis et al., 1992) and significantly more frequently than from synovial fluid of non-lame pigs. Synovial fluids must be aseptically collected during the first 3 weeks post infection for *M. hyosynoviae* isolation purposes (Hagedorn-Olsen et al., 1999a; Nielsen et al., 2001). In addition, *M. hyosynoviae* can be isolated from tonsils of pigs with and without clinical presentation (Nielsen et al., 2001), corroborating the initial hypothesis of chronic colonization of the tissue. Tonsils appear to be a more sensitive sample than nasal fluids for detection of chronic carries through culture and isolation (Ross et al., 1971; Ross and Spear, 1973).

Antibodies against *M. hyosynoviae* have been detected in previous studies by complement fixation of sera from affected pigs (Ross and Spear, 1973; Zimmermann and Ross, 1982), with detection not associated with clinical disease (Zimmermann and Ross, 1982). Humoral immunity might also be detected by different proposed ELISA's assays, including a monoclonal blocking ELISA (Pedersen et al., 1996), an indirect ELISA using freeze-thawed antigen (Kobisch and Friis, 1996), and an indirect ELISA using deoxycholate soluble antigen (Nielsen et al., 2005). The latter study suggested an association of antibody detection and tonsillar colonization, which can be inconsistent (Hagedorn-Olsen et al., 1999c), and also not associated with clinical disease. The assay proposed appeared to have low cross reactivity with *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* and higher serological responses in pigs between 12 and 20 weeks of

age (Nielsen et al., 2005). However, serology may not be suitable for individual diagnosis since pigs can be life-long carriers in tonsils (Nielsen et al., 2005), with specific antibodies against *M. hyosynoviae* being difficult to detect in early stages post experimental infection (Gomes Neto et al., 2014) and detected as far as 6 months (Zimmermann and Ross, 1982). In addition, maternal antibodies can last until 8 to 12 weeks of age (Hagedorn-Olsen et al., 1999c), although serology may aid in herd assessment.

In more recent years, *M. hyosynoviae* detection using multiplex (Stakenborg et al., 2006) and real-time PCR (Makhanon et al., 2012; Gomes Neto et al., 2015) have been proposed. Molecular detection of *M. hyosynoviae* can be achieved from joint fluids and tonsils of affected pigs (Makhanon et al., 2012; Gomes Neto et al., 2015), as well as nasal swabs and oral fluids (Gomes Neto et al., 2015). Gross and microscopic lesions on joints of affected pigs might be evaluated as supportive measure for accurate diagnosis, with techniques such as *in situ* hybridization in formalin-fixed joint tissue (Boye et al., 2001), since histological changes in affected joints might not reflect clinical disease (Hagedorn-Olsen et al., 1999a; Nielsen et al., 2001). Thus, paired sampling events and clinical sign observation is suggested for a more accurate *M. hyosynoviae* infection diagnosis (Zimmermann and Ross, 1982).

1.3.5. Control strategies

Mycoplasma hyosynoviae infections were considered sporadic events and related to specific herds. However, *M hyosynoviae* has been considered a re-emerging cause of

lameness in the swine industry due to the increased detection in field samples submitted to diagnostic laboratories for infectious agent investigation (Gomes Neto et al., 2012). However, management practices, genetics, and environmental factors have been suggested to play a role in the manifestation of mycoplasmal arthritis (Ross, 1972), thus, characterizing *M. hyosynoviae* infection as a multi-factorial process.

Due the absence of cell wall, *M hyosynoviae* is naturally resistant to β-lactams, therefore treatment with penicilins and cephalosporin is not effective. *Mycoplasma hyosynoviae* antimicrobial susceptibility has been previously reported (Aarestrup and Friis, 1998; Schultz et al., 2012), and the microorganism is suggested to be susceptible to macrolides, aminoglycosides, quinolones and tetracycline. In addition, *M. hyosynoviae* is reported to be resistant to sulfonamides (Schultz et al., 2012). However, a study evaluating the antimicrobial susceptibility of old and new strains of *M. hyosynoviae* demonstrated emerging macrolide resistance (Aarestrup and Friis, 1998). It has been suggested that the likely cause of this resistance is the widespread therapeutic use over the years (Aarestrup and Friis, 1998; Nielsen et al., 2001). In addition, it is hypothesized that early parenteral therapy results in shorter periods of lameness, although the majority of affected pigs might recover regardless of treatment (Nielsen et al., 2001).

As mentioned for *M. hyorhinis*, *M. hyosynoviae* control should also take into account effectiveness of antimicrobial treatment, especially due to the multi-factorial condition of *M. hyosynoviae* induced-disease (Ross, 1972). However, treatment decisions must always be made based on judicious use of antibiotics, animal welfare, and financial aspects, considering the undoubtful impact of *M. hyorhinis* and *M. hyosynoviae* in the

contemporary swine industry.

CHAPTER 2

Dam and piglet colonization with *M. hyorhinis* and *M. hyosynoviae* prior to weaning

2.1. Summary

The aim of this study was to characterize and compare the colonization of dams and their offspring with *M. hyorhinis* and *M. hyosynoviae* prior to weaning. A sow farm with history of lameness was selected for this study. Tonsillar swabs were collected from 29 dams of various parities and 120 piglets randomly selected from their litters at 1 and 3 weeks after farrowing. Tonsillar swabs were tested by species-specific real-time PCR to detect *M. hyorhinis* and *M. hyosynoviae* genetic material separately. One week after farrowing, *M. hyorhinis* was detected in 72% of dams and 8.3% of piglets' samples. *M. hyosynoviae* was detected in 55% of dams and none of piglets' samples. At week 3 after farrowing, *M. hyorhinis* was detected in 65% of dams and 50% of piglets' samples, and *M. hyosynoviae* was detected in 48.3% of dams and 0.9% of piglets' samples. No statistical difference was observed on dam colonization status by *M. hyorhinis* or *M. hyosynoviae*, and no association was observed between *M. hyorhinis* and *M. hyosynoviae* dam colonization status at either week of sampling. Piglet colonization with *M. hyorhinis* was significantly higher at week 3 after farrowing ($p<0.05$). Piglet colonization with *M. hyorhinis* was also significantly higher than colonization with *M. hyosynoviae* at both weeks of sampling ($p<0.05$). No association between *M. hyorhinis* and *M. hyosynoviae* piglet colonization status was observed at either week of sampling. The proportion of first parity dams with detection of *M. hyorhinis* in tonsillar swabs was significantly higher than the proportion of multiple parity dams at week 3 of sampling ($p<0.05$). Detection of *M. hyorhinis* and *M. hyosynoviae* observed in this study prior to weaning might correspond to disease presentation reported in field settings in nursery and finishing pigs.

Mycoplasma hyorhinis detection seen in this study appeared to be higher with the use of tonsillar swabs than with other sample types previously reported. This is the first investigation to evaluate *M. hyorhinis* and *M. hyosynoviae* dual colonization status of dams and their offspring prior to weaning using tonsillar swabs.

2.2. Introduction

Mycoplasma hyorhinis and *Mycoplasma hyosynoviae* are considered commensal microorganisms of the upper respiratory tract and tonsils of pigs (Ross and Spear, 1973; Gois ans Kuksa, 1974; Friis et al., 1991; Friis and Feenstra, 1994; Hagedorn-Olsen et al., 1999b). *Mycoplasma hyorhinis* is recognized as the causative agent of polyserositis and arthritis in nursery pigs (Roberts et al., 1963; Gois et al., 1977), between 8 and 10 weeks of age (reviewed by Kobisch and Friis, 1996), with infection in pigs older than 3 months of age usually resulting in mild arthritis (Potgieter and Ross, 1972). *Mycoplasma hyosynoviae* has also been proposed to be a causative agent of arthritis (Ross and Duncan, 1970), observed, however, in growing and finishing pigs (Hagedorn-Olsen et al., 1999a; Nielsen et al., 2001), with lesions being restricted to the synovial membranes (Hagedorn-Olsen et al., 1999a).

Mycoplasma hyorhinis transmission is suspected to happen from dams to piglets shortly after birth (Friis and Feenstra, 1994), and decrease after 8 weeks of age (Gois and Kuksa, 1974). In contrast, *M. hyosynoviae* is proposed as not being transmitted to pigs younger than 4-8 weeks of age (Ross and Spear, 1973). However, various investigations have reported tonsillar colonization of suckling pigs by *M. hyosynoviae* prior to weaning (Hagedorn-Olsen et al., 1999c; Schwartz et al., 2014), and there is no evidence of age restriction to *M. hyosynoviae* arthritis in experimentally infected pigs (Lauritsen et al., 2008). Nevertheless, the mechanisms of systemic spread and disease development of these two microorganisms have yet to be described.

It has been suggested that *M. hyorhinis* can persist chronically in joints (Roberts et al., 1963; Barden and Decker, 1971), and isolation of the microorganism from clinically affected animals might not be definitive for diagnosis of disease (Rovira et al., 2010; Gomes Neto et al., 2012). Similarly, *M. hyosynoviae* might be detected in joints with no apparent clinical presentation or macroscopic joint lesions observed (Ross, 1972; Nielsen et al., 2001; Nielsen et al., 2005). Colonization prevalence with *M. hyorhinis* and *M. hyosynoviae* prior to weaning has been described using several sample types and tests, such as culture of tonsil tissue and synovial fluids (Ross and Duncan, 1970; Ross and Spear, 1973; Hagedorn-Olsen et al., 1999c), serum tested by ELISA and complement fixation (Ross and Spear, 1973; Hagedorn-Olsen et al., 1999c; Nielsen et al., 2005), and nasal swabs tested by real time PCR (Clavijo, 2014). Nevertheless, *M. hyosynoviae* detection in tonsil swabs has yet to be evaluated, although tonsils scrapings have been previously investigated (Gomes Neto et al, 2015), and the tissue is considered a target for *M. hyosynoviae* colonization.

We hypothesize that dual colonization with *M. hyorhinis* and *M. hyosynoviae* can be detected in dams and piglets with the use of tonsillar swabs, and the prevalence of *M. hyorhinis* and *M. hyosynoviae* detection observed in piglets prior to weaning may be related to disease development in later production stages. However, the critical moments of piglets colonization need further examination as part of the investigation of the epidemiology of *M. hyorhinis* and *M. hyosynoviae* in swine production systems. Therefore, the aim of this study was to characterize and compare tonsillar colonization of dams and their offspring with *M. hyorhinis* and *M. hyosynoviae* prior to weaning.

2.3. Materials and Methods

2.3.1. Ethics statement

Sows and piglets were sampled following protocols approved by the University of Minnesota Institutional Animal Care and Use Committee and handled according to the sow farm standard procedures.

2.3.2. Animals and housing

A sow farm located in Minnesota, USA, composed of 2,000 sows, and with unknown *M. hyosynoviae* status at enrollment, but with history *M. hyosynoviae* clinical disease in the production system was selected for this investigation. During the study period the sow farm was negative for *M. hyopneumoniae* and endemic for porcine reproductive and respiratory syndrome virus (PRRSV).

According to the sow farm management practices, dams were vaccinated with a reproductive vaccine against porcine parvovirus, *Erysipelothrix rhusiopathiae,* and *Leptospira sp.* 3 weeks prior to farrowing and treated with a combination of lincomycin, dexamethasone and flunixin in case lameness was observed. Piglets were processed and cross-fostered if needed within 3 days of age, vaccinated at 3 days of age and one day prior to weaning against porcine circovirus type 2 (PCV2) and treated with penicillin and dexamethasone if lameness or joint edema was observed before 3 days of age. The lactation period had a duration of 21 days on average.

For this study, one hundred twenty piglets, with even distribution by gender, were randomly selected, at 3 days of age, from 29 dams of various parities, randomly selected. The sample size was estimated based on 10% expected prevalence and 95% confidence, given a population size of 2000 dams (Cannon and Roe, 1982). However, the initial sample size was not maintained due to agalactia on one of the dams.

2.3.3. Sample collection

Tonsillar swabs were collected in the farrowing rooms from dams and piglets at 1 and 3 weeks after farrowing. These sampling times were chosen according to farm management, meaning that sampling on week 1 after farrowing occurred after processing and cross-fostering of piglets, and sampling on week 3 after farrowing occurred prior to weaning.

The tonsillar swab collection process involved the use of a mouth gag and a headlamp to aid in the location of the tonsils in the mouth cavity. Tonsillar swabs were collected by rubbing a sterile swab (BBL™ CultureSwab™, Sparks, MD, USA) on the tonsils surface. The process was performed in a similar way regardless of pigs' age.

2.3.4. Sample processing and testing

Tonsillar swabs were immediately frozen and transported to the laboratory were DNA was extracted using MagMAX™-96 Viral RNA isolation kit and MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA).

A real-time PCR assay was run to detect *M. hyosynoviae* genetic material with Path-ID™ qPCR Master Mix Kit (Life Technologies, Grand Island, NY, USA),

according to manufacturer's protocol, using custom primers and probe. Primers sequence used were Forward: 5' ATA CTC TAG CGG CAA ATG GGT G 3' and Reverse: 3' CTT TCA TAA CGA AAT CAT GCG ATT T 5', and probe 5' FAM-ACC CAA TGG AAA CAT TGG TTA ATG CCG G-TAMRA 3' (University of Minnesota, Veterinary Diagnostic Laboratory; UMN-VDL). For *M. hyorhinis* genetic material detection, a real-time PCR was also run with QuantiFast Probe PCR kit (Qiagen Inc., Germantown, MD, USA), according to manufacturer's protocol, employing custom primers and probe (Clavijo et al., 2014). Samples were considered positive by real time PCR for *M. hyorhinis* and *M. hyosynoviae* when $Ct \leq 37$.

2.3.5. Statistical analysis

Detection of *M. hyorhinis* and *M. hyosynoviae* genetic material in tonsillar swabs collected from dams and piglets were compared within weeks of sampling using a two-sample test for equality of proportions. The association between *M. hyorhinis* and *M. hyosynoviae* detection in dams and piglets was evaluated by Pearson's Chi-squared test as a non-parametric 2-sample test for equality of proportions with Yates continuity correction for small sample size. A comparison of two population proportions was also performed in order to evaluate dam parity association with detection of *M. hyorhinis* and *M. hyosynoviae* in tonsillar swabs at each week of sampling. The number of dams with *M. hyorhinis* and *M. hyosynoviae* detection, *M. hyorhinis* or *M. hyosynoviae* detection only, and no detection at each week of sampling were also evaluated by Pearson's Chi-squared test. Proportions were calculated as the number of *M. hyosynoviae* detections

divided by the total number of pigs tested in that group in each week of sampling. Statistical significance was considered when *p*-values were lower than 0.05. Statistical analysis and graphical representation were performed in R v3.2 (R Core Team, 2015).

2.4. Results

Detection of *M. hyorhinis* in tonsillar swabs collected from dams and piglets tested by real time PCR is shown in Figure 1. One week after farrowing, *M. hyorhinis* was detected in tonsillar swabs of 72% (95% CI: 55.7-88.3%) of dams and 8.3% (95% CI: 3.4-13.2%) of piglets. At week 3 after farrowing, *M. hyorhinis* was detected in tonsillar swabs of 65% (95% CI: 47.6-82.4%) of dams and 50% of piglets (95% CI: 41-58.9%). The detection of *M. hyorhinis* was significantly higher in tonsillar swabs of piglets at week 3 after farrowing ($p<0.05$).

Detection of *M. hyosynoviae* in tonsillar swabs collected from dams and piglets tested by real time PCR is shown in Figure 2. One week after farrowing, *M. hyosynoviae* was detected in tonsillar swabs of 55% (95% CI: 36.9-73.1%) of dams and it was non-detected in piglets. At week 3 after farrowing, *M. hyosynoviae* was detected in tonsillar swabs of 48.3% (95% CI: 30.1-66.5%) of dams and 0.9% (95% CI: 0-2.6%) of piglets.

Detection of *M. hyorhinis* was significantly higher than *M. hyosynoviae* detection in tonsillar swabs collected from piglets at both weeks of sampling ($p<0.05$). No association between detection of *M. hyorhinis* and *M. hyosynoviae* in piglets was observed on either week of sampling. No statistical difference on detection of *M. hyorhinis* or *M. hyosynoviae*, and no association between *M. hyorhinis* and *M.*

hyosynoviae detection in tonsillar swabs collected from dams were observed on either week of sampling.

Detection of *M. hyorhinis* and *M. hyosynoviae* in tonsillar swabs collected from dams and tested by real time PCR with distribution by parity is shown in Figure 3. In week 1 after farrowing, first parity dams (P0) corresponded to 57.1% (12/21) of *M. hyorhinis* detection in tonsillar swabs and multiple parity dams (P1 or older) corresponded to 42.9% (9/21) of the total positive dams. In week 3 after farrowing, first parity dams corresponded to 73.7% (14/19) of *M. hyorhinis* detection in tonsillar swabs and multiple parity dams corresponded to 26.3% (5/19) of the total positive dams (3A). In week 1 after farrowing, first parity dams corresponded to 37.5% (6/16) of *M. hyosynoviae* detection in tonsillar swabs and multiple parity dams corresponded to 62.5% (10/16) of the total positive dams. In week 3 after farrowing, first parity dams corresponded to 50% (7/14) of *M. hyosynoviae* detection in tonsillar swabs and multiple parity dams corresponded to 50% (7/14) of the total positive dams (3B). Although, no association was observed between *M. hyorhinis* detection and parity one week after farrowing, at week 3 after farrowing, *M. hyorhinis* detection was significantly higher in tonsillar swabs collected from first parity dams when compared to multiple parity dams ($p<0.05$). No association was observed between *M. hyosynoviae* detection and parity on either week of sampling.

Paired *M. hyorhinis* and *M. hyosynoviae* detection in tonsillar swabs tested by real time PCR at each week of sampling is shown in Table 1. No difference was observed between *M. hyorhinis* and *M. hyosynoviae* detection on either week of sampling.

2.5. Discussion

This investigation aimed to evaluate the colonization of dams and piglets with *M. hyorhinis* and *M. hyosynoviae* in tonsillar swabs collected in the farrowing rooms and tested by real time PCR. Dams appeared to be consistently colonized with both *M. hyorhinis* and *M. hyosynoviae,* although piglets appeared to have higher colonization rates at week 3 after farrowing with *M. hyorhinis* compared to *M. hyosynoviae*. It was also observed that first parity dams had higher colonization rates with *M. hyorhinis* at week 3 after farrowing, yet no association between *M. hyorhinis* and *M. hyosynoviae* detection was detected.

Mycoplasma hyorhinis was detected in a higher prevalence in first parity dams than in multiple parity dams in both weeks of sampling, although this difference was not significant in week 1. Colonization with *M. hyosynoviae*, however, was more prevalent in multiple parity dams in both weeks of sampling, yet an increased in colonization was observed in first parity dams in week 3 when compared to week 1 after farrowing. The pattern of increasing prevalence between weeks 1 and 3 after farrowing observed for both microorganisms in first parity dams may reflect a more recent transmission event and consequent colonization. However, the role of first parity dams in the *M. hyorhinis* and *M. hyosynoviae* colonization dynamics has not been reported thus far. In addition, inherited variation in the tonsillar sampling process could also play a role in the observed differences in *M. hyorhinis* and *M. hyosynoviae* detection between weeks.

Mycoplasma hyorhinis was detected on piglets' samples at both weeks of sampling prior to weaning, although with significantly higher prevalence at week 3 after farrowing. In contrast, *M. hyosynoviae* was only detected on piglets' samples at week 3 after farrowing, and in a very low prevalence. Hence, these results support the hypothesis that the colonization status of piglets in lactation might influence the timing of disease development, which has been reported in nursery stage for *M. hyorhinis* (Roberts et al., 1963; Gois et al., 1977), and in finishing stage for *M. hyosynoviae* (Ross and Duncan, 1970; Hagedorn-Olsen et al., 1999a; Nielsen et al., 2001). In other words, the higher prevalence of *M. hyorhinis* detection prior to weaning, in comparison to *M. hyosynoviae,* might correspond to earlier *M. hyorhinis* disease manifestation seen in the field after weaning.

It is important to take into account that maternal derived antibodies may play a protective role against *M. hyosynoviae* colonization prior to weaning (Ross and Spear, 1973), delaying the transmission between piglets post weaning (Hagedorn-Olsen et al., 1999a), with few colonized piglets acting as carriers. *Mycoplasma hyorhinis* maternally derived antibodies decreased a month sooner than antibodies against *M. hyosynoviae* in a wean- to-finish population of pigs (Gomes Neto, 2012), which may contribute to the earlier colonization by *M. hyorhinis*. Detection of *M. hyorhinis* and *M. hyosynoviae* specific and maternally-derived antibodies was not performed in this study, since there are no serological validated tests available and results may be of challenging interpretation due to the ubiquitous characteristic of these microorganisms.

Various sample types have been employed for the detection of *M. hyorhinis* and *M. hyosynoviae* using real time PCR. Gomes Neto et al. (2015) suggested that pen-based oral fluids and tonsillar scrapings collected from post weaning pigs were capable of detecting both pathogens, however, nasal swabs were identified as a sensitive sample for *M. hyorhinis* detection only. Colonization prior to weaning in dams and piglets by *M. hyorhinis* in nasal swabs tested by real time PCR has been previously reported (Clavijo, 2014), however, prevalence lower than 10% was observed in those studies. Although only one farm was enrolled in this study, previous observations contrast to the results of this investigation and the use of tonsillar swabs, with this sample type revealing a higher proportion of detection. Interestingly, when evaluated for another swine mycoplasma, nasal swabs were reported as a sensitive sample for *Mycoplasma hyopneumoniae* detection when compared to tonsillar swabs (Sibila et al., 2004), although *M. hyopneumoniae* is not considered a commensal microorganism and is mainly observed in the respiratory tract of pigs.

Despite the diagnostic sensitivity of tonsillar swabs for detection of *M. hyorhinis* and *M. hyosynoviae* not being yet reported, a potential hypothesis is that it would fall between that of nasal swabs and tonsillar scrapings, with the later likely classified as possibly the most sensitive sample at the individual pig level (Gomes Neto et al., 2015). Due to the collection technique, tonsillar swabs may be considered a less invasive *in vivo* sample than tonsil scrapings, yet, less tonsil tissue may be harvested in the process of tonsillar swabs collection. In addition, the association between tonsillar detection and disease development needs further investigation, considering that the tonsils are a

selective tissue for *M. hyorhinis* and *M. hyosynoviae* natural colonization and may not imply disease association. Variation from the observations seen in this study is expected in different farms and systems, and sample collection can carry intrinsic result diversity.

Control of *M. hyorhinis* and *M. hyosynoviae* in the field can be challenging due to the commensal nature of these microorganisms (Ross and Spear, 1973; Gois ans Kuksa, 1974; Friis et al., 1991; Friis and Feenstra, 1994; Hagedorn-Olsen et al., 1999b), and the potential multi-factorial aspect of disease presentation associated with these species (Ross, 1972; Friis and Feenstra, 1994). Although antimicrobial treatment was being individually utilized at the sow farm, it did not eliminate prevalence in piglets prior to weaning. Consequently, *M. hyorhinis* and *M. hyosynoviae* transmission will likely continue between piglets in later production stages. Control strategies and treatment decisions in the swine industry should always be made taking into account various aspects including sensible use of antimicrobial agents and animal welfare, considering *M. hyorhinis* and *M. hyosynoviae* capacity of causing disease.

Information on *M. hyorhinis* and *M. hyosynoviae* epidemiology is sparse and efforts must be made to better understand the colonization dynamics and disease processes of these microorganisms to aid decision making by practitioners and swine producers in the application of control strategies. Although pigs seem to be colonized by both microorganisms, association between *M. hyorhinis* and *M. hyosynoviae*, and the possible competitive mechanisms of dual colonization have yet to be elucidated. To our knowledge, this is the first investigation to evaluate *M. hyorhinis* and *M. hyosynoviae* dual colonization of dams and their offspring.

2.6. Conclusions

Under the conditions of this investigation, dams appeared to be consistently colonized with both *M. hyorhinis* and *M. hyosynoviae* prior to weaning. In contrast, higher detection was observed in piglets at week 3, in comparison to week 1 after farrowing, only with *M. hyorhinis,* while colonization with *M. hyosynoviae* was remarkably minimal. In addition, high prevalence of *M. hyorhinis* on dams and piglets was obtained in this study with the use of tonsillar swabs as a sampling type. This investigation aimed to assess for the first time *M. hyorhinis* and *M. hyosynoviae* colonization of dams and their offspring prior to weaning.

Table 2.1. Paired *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* detection in tonsillar swabs collected from dams and tested by real time polymerase chain reaction (PCR) at each week of sampling after farrowing.

	Week after farrowing		*M. hyosynoviae*		
			Positive	Negative	Total
M. hyorhinis	Week 1	Positive	12	9	21
		Negative	4	4	8
		Total	16	13	29
	Week 3	Positive	11	8	19
		Negative	4	6	10
		Total	15	14	29

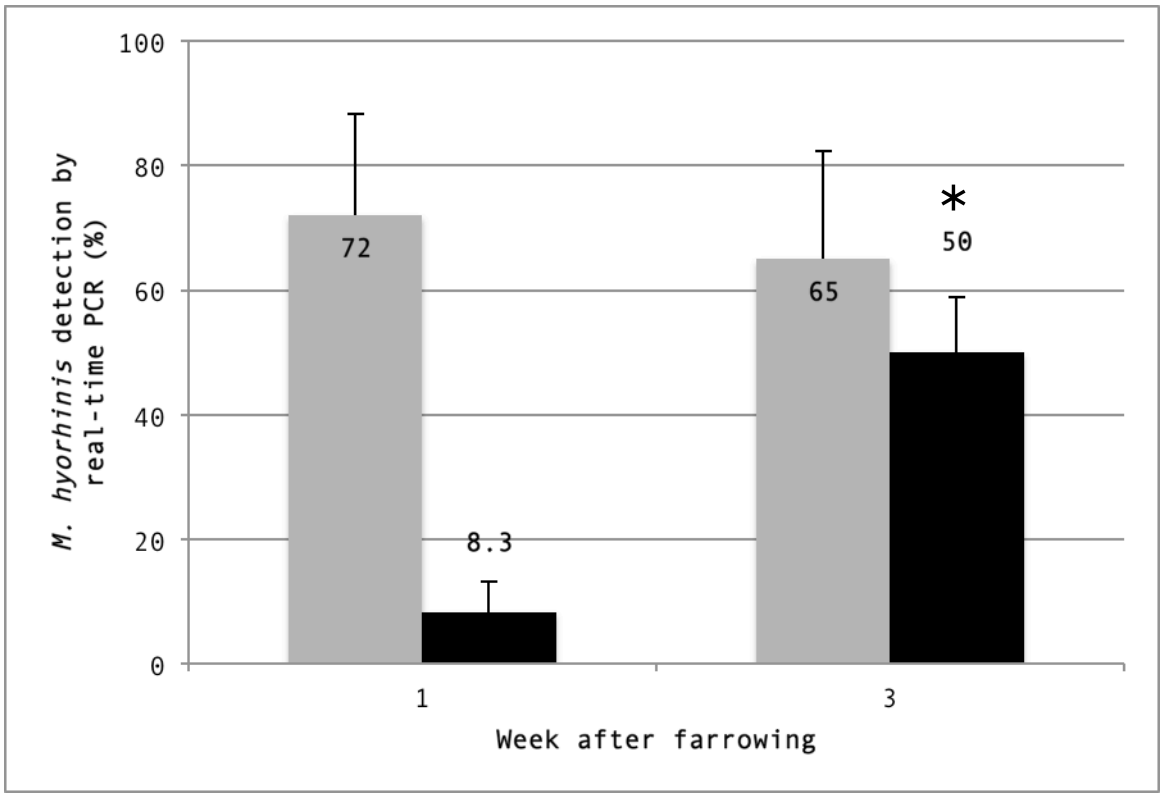


Figure 2.1. *Mycoplasma hyorhinis* detection (%; with 95% upper confidence intervals) in tonsillar swab samples collected from dams and piglets, and tested by real time polymerase chain reaction (PCR) at week 1 and 3 after farrowing. Dams (n=29/week) are represented as grey bars and piglets (n=120/week) are represented as black bars. *$p<0.05$: *M. hyorhinis* detection in piglets was significantly higher at week 3.

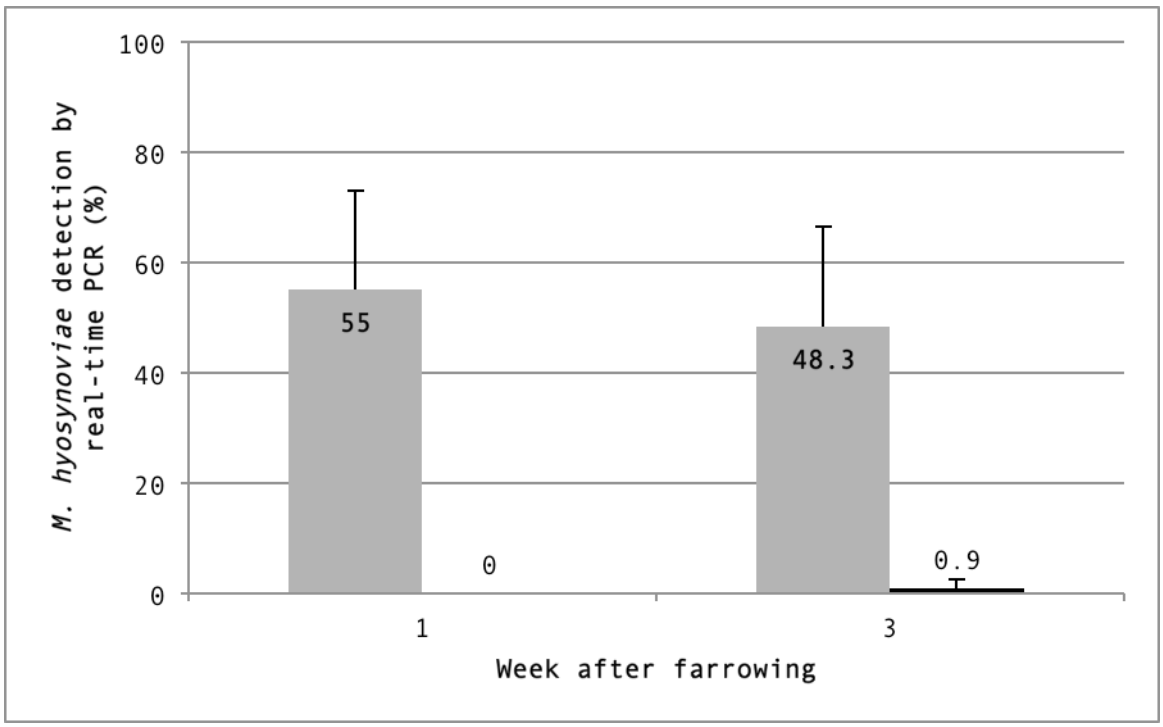
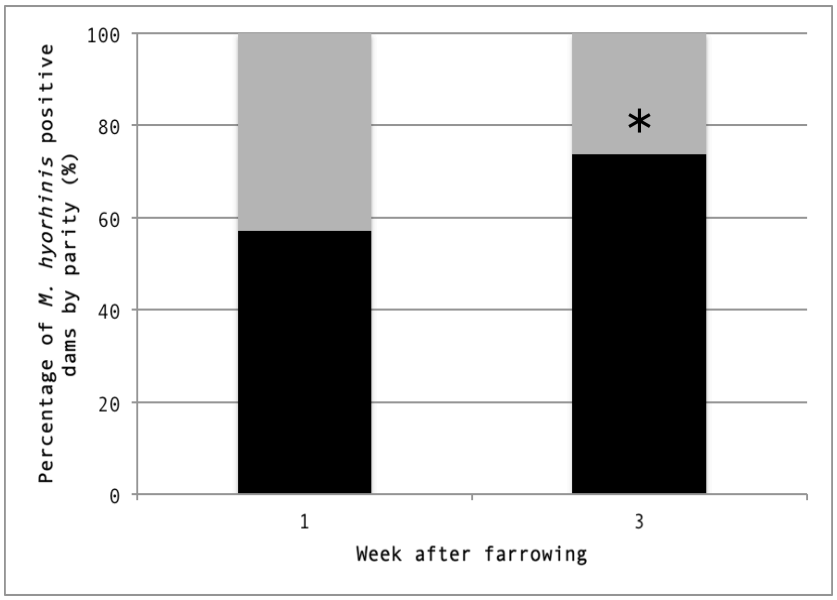


Figure 2.2. *Mycoplasma hyosynoviae* detection (%; with 95% upper confidence intervals) in tonsillar swabs collected from dams and piglets, and tested by real time polymerase chain reaction (PCR) at 1 and 3 weeks after farrowing. Dams (n=29/week) are represented as grey bars and piglets (n=120/week) are represented as black bars.

A.



B.

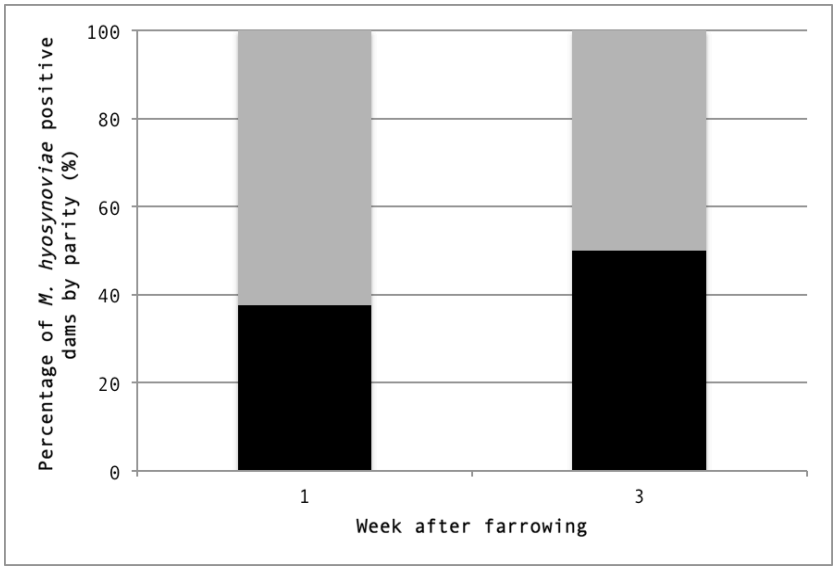


Figure 2.3. Percentage of first and multiple parity dams with detection of genetic material in tonsillar swabs by species-specific real time PCR at weeks 1 and 3 after farrowing. First parity dams are represented as black bars and sows are represented as grey bars. A) *M. hyorhinis*. In week 1 after farrowing, first parity dams (P0) corresponded to 57.1% of *M. hyorhinis* detection in tonsillar swabs and sows (P1 or older) corresponded to 42.9% of the total positive dams. In week 3 after farrowing, first parity dams corresponded to 73.7% of *M. hyorhinis* detection in tonsillar swabs and sows corresponded to 26.3% of the total positive dams. *$p<0.05$: Prevalence of *M. hyorhinis* detection in first parity dams was significantly higher at week 3 after farrowing. B) *M. hyosynoviae.* In week 1 after farrowing, first parity dams corresponded to 37.5% of *M. hyosynoviae* detection in tonsillar swabs and sows corresponded to 62.5% of the total positive dams. In week 3 after farrowing, first parity dams corresponded to 50% of *M. hyosynoviae* detection in tonsillar swabs and sows corresponded to 50% of the total positive dams.

CHAPTER 3

Mycoplasma hyosynoviae colonization and clinical presentation in growing and breeding pigs

3.1. Summary

The aim of this study was to describe the dynamics of *Mycoplasma hyosynoviae* colonization in a swine production system. At a sow farm, tonsillar swabs were collected from 29 dams and 60 piglets 1 and 3 weeks after farrowing. At a wean-to-finish barn, tonsillar swabs, oral fluids and individual and pen based lameness scores (0-4) were collected and measured from selected pigs at weeks 5, 7, 10, 13, 16, 19 and 22 of age. Hind legs from pigs that died or were euthanized were submitted for *M. hyosynoviae* detection and histopathology evaluation. At a gilt development unit (GDU), tonsillar swabs, oral fluids and individual and pen based lameness scores (0-4) were collected and measured from 55 gilts at weeks 1, 4, 8, 12 and 16 after placement. *Mycoplasma hyosynoviae* was detected in tonsillar swabs of 55% of dams at week 1, and 48.3% at week 3 after farrowing. *Mycoplasma hyosynoviae* was not detected on piglets' tonsillar swabs at the sow farm. In the wean-to-finish barn, *M. hyosynoviae* was detected in tonsillar swabs of 3.8% of pigs at week 5, 5.8% at week 7, 48.1% at week 10, 67.3% at week 13, 71.2% at week 16, 60.8% at week 19 and 82.7% at week 22 of age. In oral fluids, *M. hyosynoviae* was detected in 25% of pens at week 5, 25% at week 7, 75% at week 10, 63% at week 13, 97% at week 16 and 100% at weeks 19 and 22 of age. Joint fluid samples tested positive for *M. hyosynoviae* although lesions were not observed. At the GDU, *M. hyosynoviae* was detected in tonsillar swabs of 15% of gilts at week 1, 56% at week 4, 35% at week 8, 58% at week 12, and 75% at week 16 after placement. In gilts oral fluids, *M. hyosynoviae* was detected in 60% of pens at week 1, 80% at week 4, 100% at weeks 8 and 12, and 85% at week 16 after placement. Individual and pen based

lameness scores were low, with means not exceeding 0.5 in a 0-4 scale, and not associated with *M. hyosynoviae* tonsillar and oral fluids detection at any week in the wean-to-finish barn or the GDU. This is the first longitudinal investigation to evaluate *M. hyosynoviae* colonization in a production system using tonsillar swabs tested by real time PCR. High *M. hyosynoviae* prevalence was detected in growing and breeding pigs albeit the minimal clinical presentation observed, suggesting that *M. hyosynoviae* alone might not be competent to produce significant lameness. The results of this study indicate that association between *M. hyosynoviae* colonization and lameness, and transmission events that might lead to consequent spread of the microorganism need further investigation.

3.2. Introduction

Mycoplasma hyosynoviae is a species-specific bacterium that affects swine (Ross and Karmon, 1970) and was first recognized as arthritis inducer in the United States in 1970 by Ross and Duncan. Various studies describe *M. hyosynoviae* colonizing the respiratory tract and persisting in tonsils indefinitely as a commensal of the oral cavity and nasal passages of pigs (Ross and Spear, 1973; Friss et al., 1991). The main clinical sign observed in a *M. hyosynoviae* infection is lameness due to non-purulent polyarthritis, which is usually observed in growing and finishing pigs persisting acutely for 3 to 10 days and gradually decreasing (Hagedorn-Olsen et al., 1999a; Nielsen et al., 2001). However, it has been suggested that *M. hyosynoviae* may be present in joints without observation of clinical illness (Nielsen et al., 2001), and pigs can recover with no further consequences or a variable stiffness of motion (reviewed by Thacker, 2009). Disease morbidity varies and can reach up to 50% in affected herds, yet mortality is usually low (Ross, 1992).

The determinant factors for systemic spread of *M. hyosynoviae* and disease development have not been identified. Literature describing *M. hyosynoviae* epidemiology is sparse and the colonization patterns need to be further investigated with modern diagnostic approaches, and relevant to current production systems. It has been proposed that the microorganism is usually not transmitted to pigs younger than 4-8 weeks of age, due to lack of successful isolation from pharyngeal secretions, and is shed intermittently during the acute phase of infection (Ross and Spear, 1973). Yet, recent studies have suggested that colonization may occur in suckling pigs (Hagedorn-Olsen et

al., 1999c; Schwartz et al., 2014). Also, it has been proposed that *M. hyosynoviae* can spread throughout the pens from acutely or chronically infected pigs (Hagedorn-Olsen et al., 1999c), and not limited to transmission by acutely infected animals.

Detection of *M. hyosynoviae* can be achieved by isolation from joint fluid of affected pigs (Hagedorn-Olsen et al., 1999a), and real time PCR testing on culture (Makhanon et al., 2011), joint fluids (Gomes-Neto et al., 2012), nasal swabs, tonsil scrapings and oral fluids (Gomes-Neto et al., 2015). The use of tonsillar swabs, a non-invasive and practical *in vivo* sample, for *M. hyosynoviae* detection has yet to be evaluated, even though the tonsils are considered the target tissue for *M. hyosynoviae* colonization.

Once, *M. hyosynoviae* infections were classified as sporadic events and related to specific herds. However, *M. hyosynoviae* has been considered a re-emerging cause of lameness in the swine industry due to increasing detection in field samples submitted to diagnostic laboratories (Gomes-Neto et al., 2012), hence it requires research attention. We hypothesize that *M. hyosynoviae* early tonsillar colonization pattern might be associated with later clinical presentation. Therefore, the aim of this study was to characterize *M. hyosynoviae* colonization and lameness presentation in a swine production system using a longitudinal approach, collecting tonsillar swabs and employing molecular diagnostic techniques.

3.3. Materials and Methods

3.3.1. Ethics statement

All animals were sampled strictly following protocols approved by the University of Minnesota Institutional Animal Care and Use Committee and handled following farms specific procedures.

3.3.2. Animals and housing

A production system located in Minnesota, USA, composed of a sow farm, a wean-to-finish research barn, and a gilt development unit (GDU) barn, with history of *M. hyosynoviae* diagnosis and clinical presentation of lameness in the wean-to-finish barn and in the GDU was selected for this study. At enrollment, the sow farm was negative for *Mycoplasma hyopneumoniae* and endemic for porcine reproductive and respiratory syndrome virus (PRRSv), and the GDU was *Mycoplasma hyopneumoniae* and PRRSv negative.

3.3.3. Study design

At the sow farm, sixty 3-day old piglets, with even distribution by gender, were randomly selected from 29 dams of various parities. The sample size of 30 sows was estimated based on 10% expected prevalence and 95% confidence, given a population size of 2000 dams (Cannon and Roe, 1982), which was not maintained due to agalactia on one of the dams. After weaning, selected pigs were transported to a wean-to-finish barn and randomly distributed in pens including 7 or 8 pigs along with other pigs of the same origin. At the wean-to-finish research barn pigs were allocated to a total of 8 pens

from weeks 5 until 10. From week 11 until 22, pigs were redistributed and each pen was divided into two new pens, for a total of 16 pens in the study. By the end of the trial, the total number of pigs included in the study was 52, with 13% loss of follow up.

At the GDU barn, 55 gilts were randomly selected at 1 week after placement in the facility, at approximately 7 weeks of age. Gilts were allocated in a total of 5 pens with other gilts of same origin from week 1 until week 11 after placement. From week 12 until 16, gilts were transported to a second GDU barn and allocated into new pens with random new pen mates for a total of 13 pens. By the end of the trial, the total number of gilts included in the study was 36, with 35% loss of follow up, mainly due to non-selection.

In this study a longitudinal approach was used, meaning that piglets selected at the sow farm were followed to the wean-to-finish barn for concurrent samplings. Gilts were also followed after placement for concurrent samplings at the GDU barn.

3.3.4. Sample and data collection

At the sow farm tonsillar swabs were collected in the farrowing rooms from dams and selected piglets at 1 and 3 weeks after farrowing. The term 'after farrowing' is being used in this investigation to describe samples collected from dams and piglets at the sow farm. All selected piglets were ear-tagged at first sampling, and all dams had been previously ear-tagged by farm personnel.

After weaning and at the wean-to-finish barn, tonsillar swab collection and individual lameness scores were performed in all selected pigs at weeks 5, 7, 10, 13, 16,

19 and 22 of age. Tonsillar swabs were collected by the introduction of a sterile culture swab (BBL™ CultureSwab™, Sparks, MD, USA) into the oral cavity of the pigs, with the help of a mouth gag, until tonsils were reached and then brushed with the tip of the swab.

Oral fluids and pen based lameness scores were collected and measured from assigned pens on the same weeks as individual samplings were performed. Oral fluids collection was performed with the use of a cotton rope hung at pigs' shoulder height in the front gate of selected pens, followed by the liquid extraction from the rope with a plastic bag, transference of the liquid to a plastic tube and immediate refrigeration. Pen based lameness scores were performed by evaluating all pigs in the pen in addition to selected pigs.

At the GDU barn, tonsillar swabs collection and individual lameness score were performed in all selected gilts at weeks 1, 4, 8, 12 and 16 after placement. Oral fluids collection and pen based lameness score were performed in all assigned pens on the same weeks as individual samplings were performed.

Lameness score evaluation was performed by the same investigator throughout the study using a 0 to 4 scale proposed by Nielsen et al., 2001. Briefly, a score of 0 described a scenario were the pig gets up immediately from a lying position and moves freely in the pen with balanced weight on all four limbs. For score 1, pig rises immediately but a reluctant movement is observed, with short steps and uneven distribution of body weight. For score 2, pig moves slowly in the pen with short steps and reduced weight in the sore limb, or pig rises slowly and the affected limb was not

weight bearing. Score 3, pig is reluctant to rise, with muscle shivering when standing and lifts the sore limb from the floor, or pig refuses to walk or walks on three limbs only. Finally, for score 4, pig only rises when forced and when standing has marked signs of pain. Individual lameness scores were performed by individually evaluating selected pigs, and pen based lameness scores were performed by evaluating all pigs in the pen, as group measure, in addition to selected pigs.

3.3.5. Sample processing and testing

Tonsillar swabs and oral fluids were immediately frozen and taken to the laboratory were DNA was extracted using MagMAX™-96 Viral RNA isolation kit and MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). A real-time PCR test was run to detect *M. hyosynoviae* genetic material using Path-ID™ qPCR Master Mix Kit (Life Technologies, Grand Island, NY, USA), according to manufacturers' protocol, using custom primers and probe. Primers sequence used were Forward: 5' ATA CTC TAG CGG CAA ATG GGT G 3' and Reverse: 3' CTT TCA TAA CGA AAT CAT GCG ATT T 5', and probe 5' FAM ACC CAA TGG AAA CAT TGG TTA ATG CCG G TAMRA 3' (University of Minnesota, Veterinary Diagnostic Laboratory; UMN-VDL). Samples were considered positive for real time PCR when $Ct \leq 37$.

Hind legs collected at necropsy from selected pigs that died or were euthanized were submitted to the UMN-VDL for *M. hyosynoviae* detection by real time PCR and histopathology evaluation. Joint fluid samples were also tested for detection of

Erysipelothrix rhusiopathiae, *Haemophilus parasuis* and *M. hyorhinis* genetic material by real time PCR.

3.3.6. Data analysis

A comparison of two population proportions was performed to evaluate dam parity association with positive detection of *M. hyosynoviae* in tonsillar swabs at each week after farrowing. The relative risk of positive *M. hyosynoviae* detection at week 3 after farrowing was measured considering previous positive *M. hyosynoviae* detection at week 1 after farrowing as exposure. Proportions of positive results of individual lameness scores (score of 1 or greater) and positive detection in tonsillar swabs, as an individual measure, and proportions of positive results of pen-based lameness scores (score of 1 or greater) and positive detection in oral fluids, as a population measure, in each week of sampling were compared by Pearson's Chi-squared test as a non-parametric 2-sample test for equality of proportions with Yates continuity correction for small sample size. In addition, proportions of positive detection in tonsillar swabs on the previous or current week were compared with positive results of individual lameness scores (score of 1 or greater) on current week of each week of sampling, also by Chi-square test. Statistical significance was considered when *p*-values were less than 0.05. Proportions were calculated as the number of *M. hyosynoviae* detections divided by the total number of pigs tested in that group in each week of sampling. Statistical analysis and graphical representation were performed in R v3.2 (R Core Team, 2015).

3.4. Results

The detection of *M. hyosynoviae* in tonsillar swabs collected from dams (with distribution by parity) and their offspring prior to weaning is shown in Figure 3.1. At the sow farm, *M. hyosynoviae* was detected in tonsillar swabs of 55% (95% CI: 36.9-73.1%) dams at week 1 after farrowing, with first parity dams (P0) corresponding to 37.5% and multiple parity dams (P1 or older) corresponding to 62.5% of detection, while no detection was obtained in tonsillar swabs collected from piglets. At week 3 after farrowing, *M. hyosynoviae* was detected in tonsillar swabs of 48.3% (95% CI: 30.1-66.5%) of dams, with first parity dams corresponding to 50% and multiple parity dams corresponded to 50% of detection, and remained undetected in tonsillar swabs collected from piglets. No association between *M. hyosynoviae* detection and parity was observed at any week of sampling ($p>0.05$).

Individual *M. hyosynoviae* detection in tonsillar swabs collected from dams at each week after farrowing is shown in Figure 3.2. Thirty four percent of dams were colonized with *M. hyosynoviae* in tonsillar swabs in both weeks of sampling, 21% of dams at week 1, 14% of dams at week 3, and 31% of dams in neither week of sampling. Considering *M. hyosynoviae* detection results at both weeks in tonsillar swabs collected from dams, the estimated relative risk of positive detection in week 3 after farrowing was 1.78 (95% CI: 0.88-3.6). In other words, dams with positive detection at week 1 were 1.78 times at risk of positive detection at week 3 compared to dams with no detection at week 1, although these differences were not statistically significant ($p>0.05$).

The detection of *M. hyosynoviae* in tonsillar swabs collected from pigs at the wean-to-finish research barn is shown in Figure 3.3. Briefly, *M. hyosynoviae* was detected in tonsillar swabs of 3.8% (95% CI: 0-9%) of pigs at week 5, 5.8% (95% CI: 0-12.1%) at week 7, 48.1% (95% CI: 34.5-61.7%) at week 10, 67.3% (95% CI: 54.5-80.1%) at week 13, 71.2% (95% CI: 58.9-83.5%) at week 16, 60.8% (95% CI: 47.5-74.1%) at week 19 and 82.7% (95% CI: 72.4-93%) at week 22 of age. In oral fluids, *M. hyosynoviae* was detected in 25% (95% CI: 0-55%) of pens at weeks 5 and 7, 75% (95% CI: 44.9-100%) at week 10, 63% (95% CI: 39.3-86.7%) at week 13, 97% (95% CI: 88.6-100%) at week 16 and 100% at weeks 19 and 22 of age. Mean individual and pen based lameness scores measured at the wean-to-finish research barn are shown in Table 3.1.

Histopathology of joint tissue of a non-lame pig showed no evidence of lesion, however *M. hyosynoviae* genetic material was detected by real time PCR. No genetic material of *E. rhusiopathiae*, *H. parasuis* or *M. hyorhinis* was detected.

No association was observed between individual lameness scores observed and real time PCR testing in tonsillar swabs at any week of age in the wean-to-finish barn. In addition, no association was observed between pen-based lameness scores and real time PCR detection in oral fluids at any week of age. At previous and current week comparison, a significant association ($p<0.05$) was observed between positive detection of *M. hyosynoviae* by real time PCR in tonsillar swabs at previous week and positive results of individual lameness scores at current week between weeks 7 and 10 of age. However, the association was in an opposite direction than expected. In other words, a decrease in individual lameness scores and a significant ($p<0.05$) increase in *M.*

hyosynoviae detection by real time PCR in tonsillar swabs were observed between weeks 7 and 10 of age.

Detection of *M. hyosynoviae* in tonsillar swabs and oral fluids collected at the GDU barn is shown in Figure 3.4. At the GDU barn, *M. hyosynoviae* was detected in tonsillar swabs of 15% (95% CI: 5.6-24.4%) of gilts at week 1, 56% (95% CI: 42.5-69.5%) at week 4, 35% (95% CI: 22-47.9%) at week 8, 58% (95% CI: 42.3-73.7%) at week 12, and 75% (95% CI: 60.9-89.1%) at week 16 after placement. *Mycoplasma hyosynoviae* detection in tonsillar swabs collected from gilts was significantly higher ($p<0.05$) at week 4 after placement. In gilt oral fluids, *M. hyosynoviae* was detected in 60% (95% CI: 17.1-100%) of pens at week 1, 80% (95% CI: 44.9-100%) at week 4, 100% at weeks 8 and 12, and 85% (95% CI: 65.6-100%) at week 16 after placement. Mean individual and pen based lameness scores measured at the GDU barn are shown in Table 3.2.

No association was observed between individual lameness scores and real time PCR detection in tonsillar swabs at any week of sampling at the GDU barn. In addition, no association was observed between pen-based lameness scores and real time PCR detection in oral fluids at any week of sampling at the GDU barn.

3.5. Discussion

This investigation was designed to identify the colonization pattern of *M. hyosynoviae* in dams and their offspring prior to weaning by real time PCR in tonsillar swabs and in wean-to-finish pigs and replacement gilts using tonsillar swabs and oral

fluids. In addition, the association between *M. hyosynoviae* colonization and clinical presentation of lameness in wean-to-finish and GDU barns was evaluated. The initial hypothesis that *M. hyosynoviae* early tonsillar colonization may be associated with clinical presentation observed in later production stages could not be corroborated. Hence, in this study, prevalence *M. hyosynoviae* detection was not associated with lameness observed in breeding and finishing pigs.

At the sow farm, dams appeared to be markedly colonized with *M. hyosynoviae* prior to weaning, consistent with previous literature (Ross and Spear, 1973), although parity was not associated with *M. hyosynoviae* detection. In piglets, however, *M. hyosynoviae* was not detected in any of tonsillar swabs collected prior to weaning, as previously described (Ross and Duncan, 1970), possibly due to the exploratory sample size based on 10% expected prevalence used in this study. Piglet colonization by *M. hyosynoviae* needs further investigation for the identification of the transmission events between dams and piglets, since it has been reported that colonization of suckling pigs may occur prior to weaning (Hagedorn-Olsen et al., 1999c; Schwartz et al., 2014) and observed tonsillar colonization was intermittently detected in dams in this study. No age-related susceptibility restriction to *M. hyosynoviae* colonization has been reported (Lauritsen et al., 2008), although it has been suggested that maternal antibodies might last until 8 to 12 weeks of age (Hagedorn-Olsen et al., 1999c), possibly playing a protective role prior to weaning (Ross and Spear, 1973). However, antibody response to *M. hyosynoviae* was not measured in this study.

At the wean to finish barn, week 10 of age was identified as the onset of *M. hyosynoviae* spread with closely 50% of pigs detected positive in tonsillar swabs, despite the lack of clinical lameness, which was not associated with colonization in an individual or pen-based level. It has been proposed that the colonization pattern post-weaning tends to be inconsistent and variable between individual pigs, with carrier stage not being achieved before 8 weeks of age (Hagedorn-Olsen et al., 1999c). The significant difference observed in the relationship between tonsillar swab detection and clinical signs outcome at weeks 7 and 10 of age might be related to various possible factors and agents that can cause arthritis post weaning, such as *M. hyorhinis* (Roberts et al., 1963) and *H. parasuis* (reviewed by Rapp-Gabrielson et al., 2009), as well as transportation fallout, which were not evaluated in this investigation.

Clinical signs observed at the wean-to-finish barn were lenient, with basal levels of lameness scores recorded in individual and pen-based instances. Lameness is identified as a multifactorial process with several environmental aspects and different pathogens possibly playing a role in the development of the clinical condition associated with pain (Jensen et al., 2012), and clinical signs observation can be subjective. In addition, the study was performed in a wean-to-finish research barn, which could have masked the multifactorial environment of a commercial farm. However, findings on the joint tissue were compatible with the presence of *M. hyosynoviae* genetic material although no lesions were observed and no other pathogens were identified. It has been recommended that confirmation of *M. hyosynoviae*-associated arthritis require detection of the pathogen in the affected joints and observation of the microscopic lesions in joint tissue (Gomes-

Neto et al., 2012). Nevertheless, a clear case definition of *M. hyosynoviae* lameness is not available, therefore it was not used in this study, and collection of synovial fluids *in vivo* may be more informative in future investigations.

At the GDU barn, gilts appeared to be placed already colonized with the microorganism, consistent with observations at the wean-to-finish and previously reported (Hagedorn-Olsen et al., 1999c), since gilts were approximately 7-8 weeks of age at placement. At week 12 after placement, gilts were transferred to a different farm and mixed with pigs of various unknown origins, and at week 18, the farm became PRRSv positive. These factors might have influenced the results of clinical sign observation since trauma can occur during transportation and mixing (Nakano and Aherne, 1988; Sutherland et al., 2009), and lameness might be observed in PRRSv infected pigs, due to infection by secondary bacteria (reviewed by Dewey, 2009). Mixing with different gilts not part of the study might have also resulted in lower sensitivity of the oral fluids, not reflecting the actual colonization status of selected gilts through this sample.

In vivo samples previously suggested for detection of *M. hyosynoviae* genetic material include nasal swabs, tonsil scrapings and oral fluids (Gomes-Neto et al., 2012; Gomes-Neto et al., 2015), with tonsillar scrapings being reported as usually more sensitive for *M. hyosynoviae* detection than nasal swabs (Gomes-Neto et al., 2015) by real time PCR in individual pigs. In this study, tonsillar swabs were selected as individual samples due to its practicality and safety, implying that this sample can be potentially used in field settings by practitioners, albeit diagnostic sensitivity has yet to be evaluated.

To our knowledge, this is the first investigation using tonsillar swabs as elective *in vivo* sample for *M. hyosynoviae* detection.

Although the pathogenicity of *M. hyosynoviae* has been established since the 70s, clinical signs outcome seems to vary greatly among different strains (Ross, 1973; Nielsen et al., 2001; Gomes Neto et al 2016). In addition, in case of an outbreak, multiple strains might be involved (Kokotovic et al., 2002b). It is also important to consider the variation of colonization and infection processes across different production systems, and observations seen in this study may not reflect what is detected in different farms. However, data generated in this study can be a valuable tool in the identification of critical moments for prevention or control of *M. hyosynoviae* spread in a swine production system. Moreover, mechanisms of *M. hyosynoviae* transmission and spread need further exploration to aid the understanding of the epidemiology of the microorganism, since the moment and circumstances when pigs become colonized are still undetermined.

3.6. Conclusions

Under the conditions of this study, *M. hyosynoviae* was intermittently detected in dams but not in piglets' tonsillar swabs at the sow farm. At the wean-to-finish barn, week 10 of age appeared to be the critical moment of spread of *M. hyosynoviae* since it was detected on tonsillar swabs of approximately 50% of pigs, a significant increase from week 7 of age. In GDU barns, gilts seemed to be placed already colonized with *M. hyosynoviae.* No association between detection of *M. hyosynoviae* genetic material and

clinical outcome of lameness was observed in wean-to-finish and GDU barns, although findings in joint tissue samples were compatible with the presence of *M. hyosynoviae* genetic material. The results of this study indicate that further investigation is needed for the identification of association between *M. hyosynoviae* colonization and lameness, as well as transmission events that might lead to consequent spread of the microorganism in the herd. Such knowledge will be essential in the advancement of *M. hyosynoviae* disease control measures.

Table 3.1. Mean individual and pen based lameness scores (0 to 4 scale; Nielsen et al., 2001) observed in different weeks of age at the wean-to-finish research barn. Means do not exceed 0.5 in a 0-4 scale due to the small number of animals with score of 1 or greater. Individual lameness scores were performed by individually evaluating selected pigs, and pen based lameness scores were performed by evaluating all pigs in the pen, as group measure, in addition to selected pigs.

	Mean lameness score	
Week of age	Individual	Pen based
5	0.228	0.048
7	0.214	0.062
10	0.089	0.032
13	0.075	0.025
16	0.056	0.012
19	0.075	0.031
22	0.134	0.081

Table 3.2. Mean individual and pen based lameness scores (0 to 4 scale; Nielsen et al., 2001) observed in different weeks of sampling after placement at the gilt development unit (GDU) barn. Means do not exceed 0.5 in a 0-4 scale due to the small number of animals with score of 1 or greater. Individual lameness scores were performed by individually evaluating selected pigs, and pen based lameness scores were performed by evaluating all pigs in the pen, as group measure, in addition to selected pigs.

	Mean lameness score	
Week of sampling	Individual	Pen based
1	0.13	0.0564
4	0	0.0244
8	0	0.0418
12	0	0
16	0.083	0.004

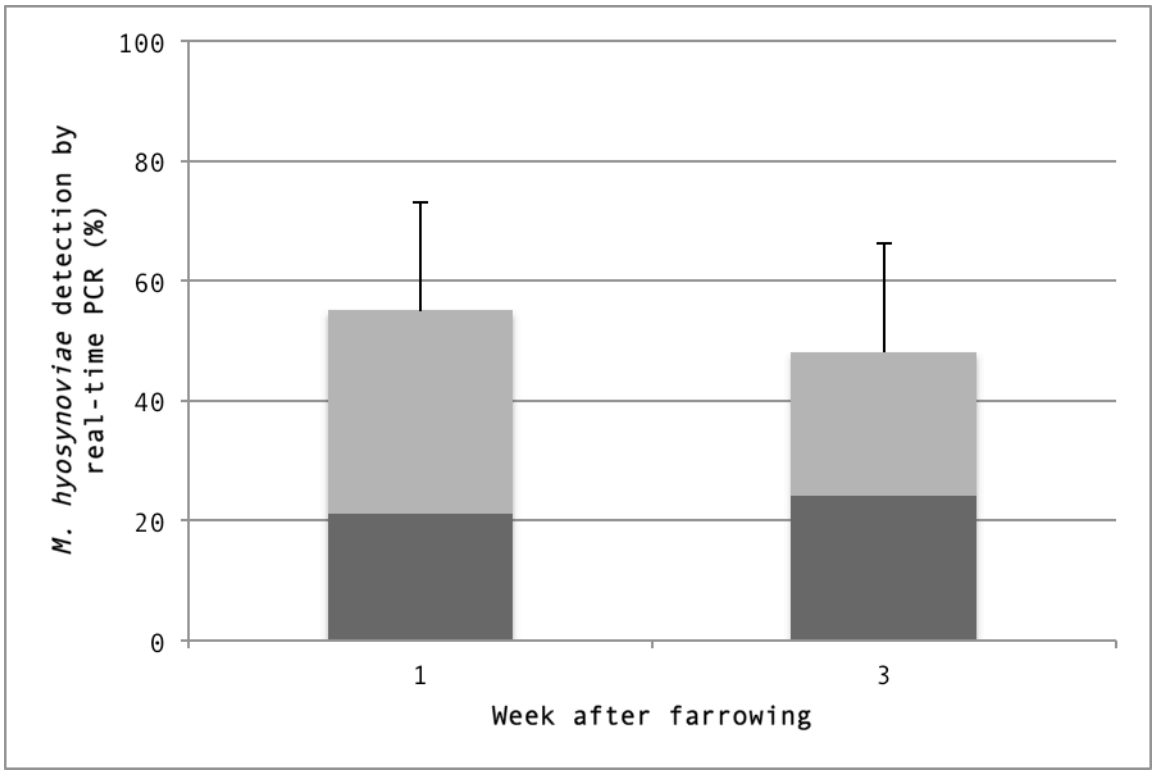


Figure 3.1. *Mycoplasma hyosynoviae* detection (%; with 95% upper confidence intervals) in tonsillar swabs of dams, and piglets tested by real time PCR at week 1 and 3 after farrowing. Dams are represented as grey bars (first parity dams: dark grey; multiple parity dams: light grey) and piglets are represented as black bars. In week 1 after farrowing, first parity dams (P0) corresponded to 37.5% *M. hyosynoviae* detection and multiple parity dams (P1 or older) corresponded to 62.5%. In week 3 after farrowing, first parity dams corresponded to 50% *M. hyosynoviae* detection and multiple parity dams corresponded to 50%.

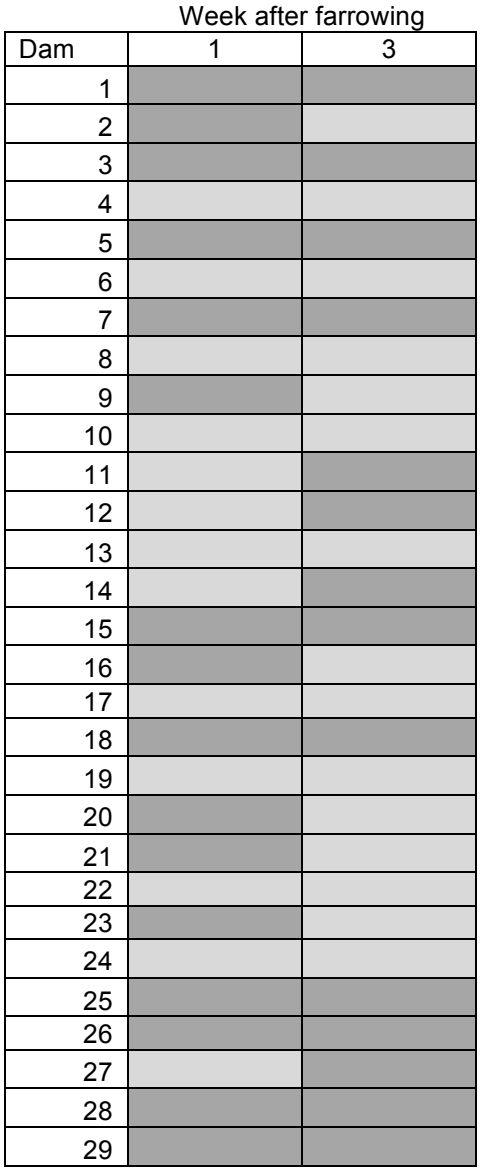


Figure 3.2. Individual *Mycoplasma hyosynoviae* detection in tonsillar swabs collected from dams at weeks 1 and 3 after farrowing. Positive *M. hyosynoviae* detection by real time polymerase chain reaction (PCR) is represented in dark grey and no detection is represented in light grey.

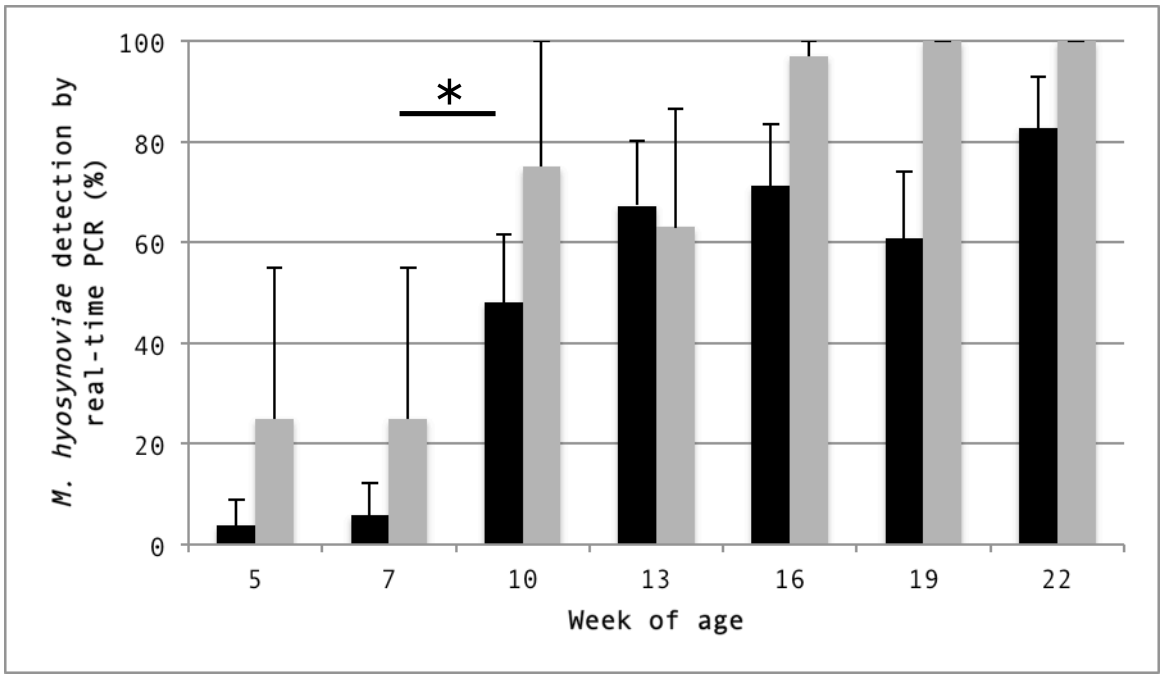


Figure 3.3. *Mycoplasma hyosynoviae* detection (%; with 95% upper confidence intervals) in tonsillar swabs and oral fluids of pigs at the wean-to-finish research barn tested by real time polymerase chain reaction (PCR) in different weeks of age. Tonsillar swabs (n~60/week) are represented as black bars and oral fluids are represented as grey bars. *p<0.05: *M. hyosynoviae* detection in tonsillar swabs and oral fluids was significantly higher at week 10 of age.

Pig	Week 5	Week 7	Week 10	Week 13	Week 16	Week 19	Week 22
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
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Figure 3.4. Individual *Mycoplasma hyosynoviae* detection in tonsillar swabs collected from piglets between weeks 5 and 22 of age. Positive *M. hyosynoviae* detection by real time polymerase chain reaction (PCR) is represented in dark grey and no detection is represented in light grey. Lameness score (Nieslen et al., 2001) of 1 or greater is represented by crossed bars.

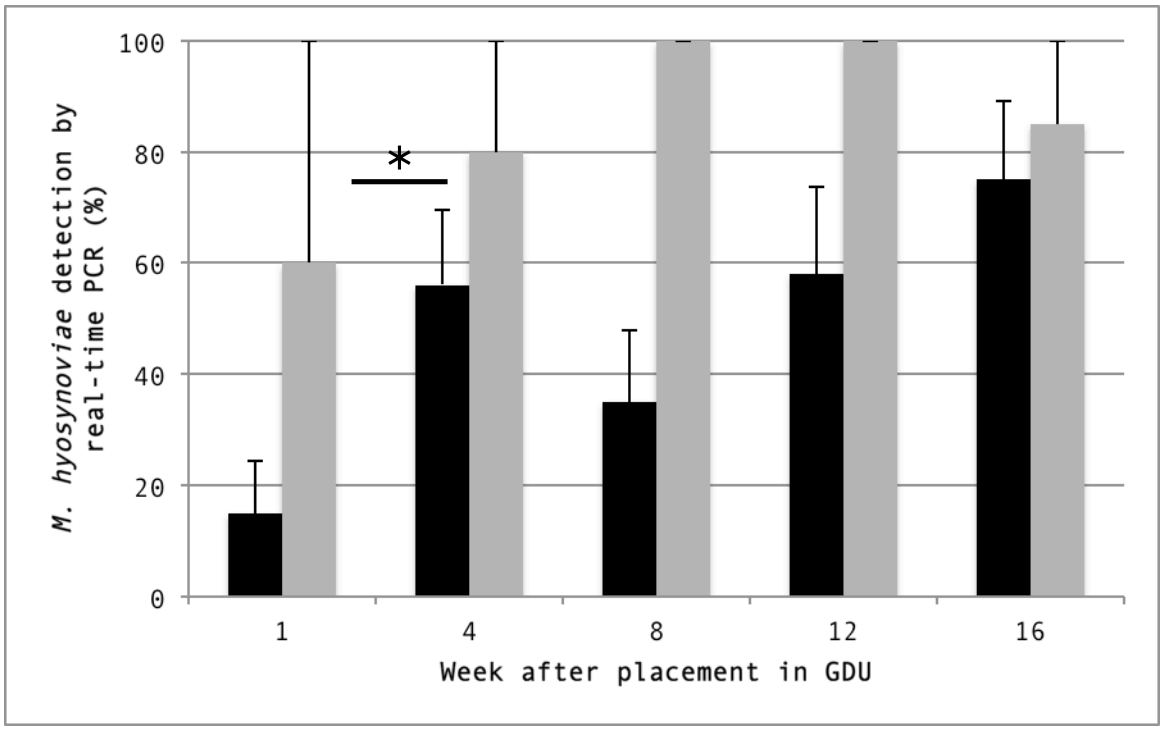

Figure 3.5. *Mycoplasma hyosynoviae* detection (%; with 95% upper confidence interval) in tonsillar swabs and oral fluids of gilts tested by real time polymerase chain reaction (PCR) in different weeks of sampling after placement at the gilt development unit (GDU) barn. Detection in tonsillar swabs (n~55/week) is represented as black bars and in oral fluids is represented as grey bars. *p<0.05: *M. hyosynoviae* detection in tonsillar swabs was significantly higher at week 4 after placement.

Gilt	Week 1	Week 4	Week 8	Week 12	Week 16
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
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42					
43					
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45					
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47					
48					
49					
50					
51					
52					
53					
54					
55					

Figure 3.6. Individual *Mycoplasma hyosynoviae* detection in tonsillar swabs collected from gilts between weeks 1 and 18 after placement at the gilt development unit (GDU). Positive *M. hyosynoviae* detection by real time polymerase chain reaction (PCR) is represented in dark grey and no detection is represented in light grey. White boxes represent missing information due to loss to follow up. Lameness score (Nielsen et al., 2001) of 1 or greater is represented by crossed bars.

GENERAL DISCUSSION

Mycoplasmas that affect swine appear to have distinct capacities and levels of disease severity, likely due to diverse tissue tropism, mechanism of disease, and inherited virulence within different species and strains. *Mycoplasma hyorhinis* is noticed as the first swine mycoplasma to grow in culture, becoming predominant in the mycoplasmal disease investigation. However, *M. hyorhinis*-induced disease is not observed *in vivo* as the most economically significant in pigs, and other mycoplasmas are responsible for a more severe disease process. Pathogenic capabilities and clinical disease have been demonstrated, however, the assessment and diagnosis of disease caused by either *M. hyorhinis* or *M. hyosynoviae* are still challenging, presumably due to their commensal nature and to the knowledge gap on the pathogenesis and epidemiology of both microorganisms. Therefore, the present series of studies focused on investigating the colonization patterns of *M. hyorhinis* and *M. hyosynoviae* by evaluating the prevalence and potential association of the dual *M. hyorhinis* and *M. hyosynoviae* colonization status of dams and piglets prior to weaning, and *M. hyosynoviae* in growing and breeding pigs.

In chapter 1, literature describing *M. hyorhinis* and *M. hyosynoviae* etiology, pathogenesis and transmission was reviewed. It is forthwith the recognition of the distinctly larger body of literature available on *M. hyorhinis* etiology and identification when compared to transmission and control aspects. The specific studies on the *M. hyorhinis* variable surface lipoprotein (Vlp) system, for example, were essentially motivated as a result of its distinct characteristic as a cell culture contaminant in human studies. Seemingly, information generated on *M. hyosynoviae* is markedly limited, with hardly 2 manuscripts published per year since it was first described in the seventies. In

essence, a knowledge deficiency is often encountered, and additional data is called for regarding *M. hyorhinis* and *M. hyosynoviae* potential implications in the swine industry.

In chapter 2, tonsillar swabs were employed for the molecular detection of *M. hyorhinis* and *M. hyosynoviae* in pigs in the farrowing rooms. Although piglets seemed to be colonized with *M. hyorhinis* only at the end of the lactation period, dams appeared to be consistently colonized by both *M. hyorhinis* and *M. hyosynoviae* throughout the lactation period. Detection of *M. hyorhinis* in dams appeared to be higher with the use of tonsillar swabs than with previous reports using nasal swabs, suggesting the diagnostic potential of this sample. The observed colonization pattern in piglets may reflect the *M. hyorhinis* and *M. hyosynoviae* clinical disease presentation observed in growing and finishing pigs, as well as the *in vitro* growth behavior of *M. hyorhinis*. However, the number of dams and piglets sampled in this study was exploratory, meaning that prevalence of *M. hyorhinis* and *M. hyosynoviae* in pigs in the production system prior to this investigation was unknown, and might not be the recommended sample size to extrapolate the observed results to the entire herd. In addition, diagnostic sensitivity of tonsillar swabs has not yet been reported, limiting the comparison with other validated samples for *M. hyorhinis* and *M. hyosynoviae* detection. Another limitation faced is this study is related to maternal derived antibodies that can limit or delay *M. hyorhinis* and *M. hyosynoviae* colonization in piglets. Specific antibody production by dams was not measured in the study, mainly due to the absence of commercially available assays and the nature of this investigation as preliminary. Nevertheless, the mechanisms of dual colonization, including disease mechanisms and immunity, and possible competitiveness

between *M. hyorhinis* and *M. hyosynoviae* in tonsils, and its consequences in disease development have yet to be investigated.

In chapter 3, pigs were evaluated longitudinally from farrow to finish, and after placement at the gilt development unit, for the investigation of *M. hyosynoviae* colonization and clinical disease presentation. *Mycoplasma hyosynoviae* spread in wean-to-finish pigs was identified as week 10 of age, and gilts appeared to be already colonized with *M. hyosynoviae* at placement in the gilt development unit. Although high detection rates were observed in wean-to-finish and breeding pigs, no association was observed between *M. hyosynoviae* longitudinal colonization and lameness. Different microorganisms that may produce similar clinical outcomes in wean-to-finish pigs were not measured *in vivo,* being limited to the *post mortem* investigation in later stages. Likewise, the origin of the decrease in lameness observed at week 7 of age was not investigated, although it seemed to be related to causes other than *M. hyosynoviae*. In addition, lameness observed in pigs close to market age might also occur due to osteochondrosis, a non-infectious process seen in fast growing animals, which was not measured in this study. In summary, systemic spread of *M. hyosynoviae* is yet to be unveiled, not only its mechanisms but also underlying conditions or predisposing factors that lead to clinical disease need further investigation. These results supplement the paradigm of *M. hyosynoviae* role in swine production systems, since colonization is largely observed in spite of the lack of clinical disease.

In contrast to *M. hyopneumoniae*, a swine pathogen of major significance associated with enzootic pneumonia and the porcine respiratory disease complex,

information about *M. hyorhinis* and *M. hyosynoviae* epidemiology is scarce, and the means of transmission and circumstances where pigs become colonized need to be further evaluated. Transmission between dams and piglets is observed prior to weaning, although the starting points of these events have yet to be explored. An intriguing aspect about these mycoplasmas is the fact that the majority of colonized pigs do not seem to develop disease, and the path between the occurrence of natural transmission and clinical disease in field settings is unknown. Recent studies have pursued better understanding of molecular evolution and variation, and possible virulence factors that might be involved with disease expression, although little is known about mechanisms of immune evasion. It has been hypothesized that many factors may play a role in the *M. hyorhinis* and *M. hyosynoviae* disease development, however, most of these factors, such as genetics and environment, are difficult to assess and vary greatly between systems. Nonetheless, disease caused by *M. hyorhinis* and *M. hyosynoviae* can contribute to compromised welfare of pigs and increased production costs, and epidemiological aspects that can aid the improvement of control measures require further research.

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