Diagnostic and field investigations in *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis*

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Microbiology

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2012

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DEDICATION

I dedicate this dissertation to my wonderful wife Renata Spuri Gomes who has been incredibly patient with me along the way. To my parents, brother and sister, aunts and uncles, and my blessed grandmothers who have supported me with love and through our conversations. To my friend and life mentor Marcos Horacio Rostagno for everything he has done to help me since my first thought about pursuing my graduate degree; otherwise, I am sure I would not be here today. Last, but not least, to all my friends and colleagues who have helped me at critical points in my life.
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ABSTRACT

Swine mycoplasmas are acknowledged for their potential to contribute to several swine disease complexes. In addition, mycoplasma-associated arthritis has recently been recognized as a re-emergent issue in the swine industry. Reports from Veterinary Diagnostic Laboratories suggest an increasing incidence of cases occurring in the major regions of swine production in the U.S. Although previous studies reproduced polyarthritis by inoculating pigs with either *Mycoplasma hyosynoviae* or *M. hyorhinis*, there is a lack of information regarding the epidemiology of these microorganisms within swine populations. In addition, although new diagnostic tools have become available, information regarding the application and performance of these assays is lacking.

Therefore, a major objective of the work described in this thesis was to review and to provide new information regarding the use of diagnostic methods in the detection of *M. hyosynoviae* and *M. hyorhinis*. In order to evaluate the performance of a new real-time polymerase chain reaction (RT-PCR), a comparison of various DNA extraction methods using different clinical specimens was conducted in the laboratory. Thereafter, a field study was carried using RT-PCR- and antibody-based assays to monitor the infection dynamics of these microorganisms in a wean-to-finish population.
THESIS ORGANIZATION

The thesis begins with a review of the literature concerning mycoplasma-associated arthritis. In particular, the review focused on *M. hyosynoviae* and *M. hyorhinis*, including pathogenesis, clinical signs, animal selection, diagnostic sample collection, and diagnostic assays to be used for mycoplasma-associated arthritis cases. The review was published in the *Journal of Swine Health and Production*.

In Chapter 2, diagnostic methods were evaluated by comparing the rate of detection of *M. hyosynoviae* or *M. hyorhinis* by real-time polymerase chain reaction (RT-PCR) in clinical specimens, i.e., oral fluids (n = 60), tonsil scraping fluids (n = 49), nasal swab fluids (n = 81), and serum (n = 150), collected from pigs selected from 5 commercial finisher sites. Nucleic acid extraction methods compared in the study included high volume (HV) and low volume (LV) extraction using magnetic beads and a spin column-based protocol (SC).

In Chapter 3, the dynamics of *M. hyosynoviae* and *M. hyorhinis* infections were followed over time in a wean-to-finish population using RT-PCR- and antibody-based assays.

The thesis ends with General Conclusions discussing the major findings of the research and the author's observations and opinions regarding the application of these new techniques in the diagnosis and field surveillance of *M hyosynoviae* and *M. hyorhinis*. 
CHAPTER 1. MYCOPLASMA-ASSOCIATED ARTHRITIS: CRITICAL POINTS FOR DIAGNOSIS

Published in the Journal of Swine Health and Production

Joao Carlos Gomes Neto; Phillip C. Gauger; Erin L. Strait; Neil Boyes; Darin M. Madson; Kent J. Schwartz

Abstract

*Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* are known causative agents of arthritis in post-weaned swine. Data from the Iowa State University Veterinary Diagnostic Laboratory shows that diagnosis of *M. hyosynoviae*- and *M. hyorhinis*-associated arthritis has increased in swine in the Midwest United States. This diagnostic note summarizes disease characteristics of both pathogens and describes appropriate procedures to diagnose mycoplasma-associated arthritis. An accurate diagnosis is critical to establishing effective treatment and prevention measures in affected herds.

**Keywords**: swine, arthritis, *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae*.

**Introduction**

Arthritis is defined as inflammation of the intra-articular tissue of one or more joints. It is characterized by an increased volume of intra-articular fluid with specific features that vary depending on the cause. Features that may have diagnostic significance include color, turbidity, hemorrhage, or exudate. In addition, arthritis may be classified according to the cause, the duration (i.e., acute, chronic), or the components of the exudate, i.e., serous, fibrinous, purulent, macrophagic, lymphoplasmacytic (Weisbrode et al., 2001).

Infectious arthritis in swine is commonly associated with bacteria. Pathogens isolated from arthritic joints may include *Erysipelothrix rhusiopathiae*, *Streptococcus suis*, *Haemophilus parasuis*, *Actinobacillus suis*, *Arcanobacterium pyogenes*, *Staphylococcus* species, *Salmonella choleraesuis*, *Mycoplasma hyorhinis*, and *Mycoplasma hyosynoviae* (Friis et al.,...
Infectious arthritis is an increasing concern to swine producers as it may contribute to compromised animal welfare and decrease profitability of the operation. Despite concerns and a long history of recognition, specific information is often lacking regarding the pathogenesis, epidemiology, and control of infectious arthritis in modern swine-production systems.

The Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL) diagnosed 431 cases of lameness between 2003 and 2010. Overall, 69% (298 of 431) of the clinical cases had evidence of infectious arthritis. Mycoplasma species accounted for an average of 17% of arthritis cases over all 8 years, with an increasing frequency in recent years, up to 37% (41 of 111) in 2010 (Figure 1). *Mycoplasma hyorhinis* was diagnosed more frequently in animal ≤ 10 weeks of age. Conversely, *M. hyosynoviae* was diagnosed more often in animals > 10 weeks of age (Figure 2). However, due to the inherent bias of diagnostic data and lack of random sampling, it is difficult to correlate these numbers to the actual disease prevalence in the field.

In response to the increasing number of mycoplasma-associated arthritis cases diagnosed at the ISU-VDL, this article will describe disease characteristics as well as proper sampling and diagnostic tests necessary to establish a diagnosis of *M. hyorhinis* - and *M. hyosynoviae-* associated arthritis in swine.

**Pathogenesis**

*Mycoplasma hyorhinis* colonizes the upper respiratory tract of pigs, and may be isolated from swine lungs with or without pneumonia (Kobisch et al., 1996; Lin et al., 2006). However, systemic disease is more commonly diagnosed after weaning (Thacker, 2006). The process of systemic dissemination remains unknown, but affinity for serosal surfaces may lead to acute inflammation of the serosa of body cavities and synovium (membrane lining the joint cavity). If not effectively mitigated by the immune response or medication, infections at those sites may become chronic, resulting in compromised growth and performance.
Serofibrinous pleuritis, pericarditis, and peritonitis are typical lesions that may generate fibrous adhesions in chronic stages (Kobisch et al., 1996; Magnusson et al., 1998; Thacker, 2006).

*Mycoplasma hyosynoviae* may cause arthritis in pigs approximately 2 to 3 weeks post exposure (Hagdorn-Olsen et al., 1999a). Tonsils are the primary site of infection, which may persist to the adult age and occasionally cause systemic disease at that time (Hagdorn-Olsen et al., 1999a; Hagdorn-Olsen et al., 1999b; Kobisch et al., 1996; Thacker, 2006). There is an incubation period of approximately 3 to 10 days that may often follow stress, such as movement or vaccination (Hagdorn-Olsen et al., 1999a; Hagdorn-Olsen et al., 1999b; Thacker, 2006). The acute phase of infection lasts approximately 2 weeks, during which time the pathogen may spread to the joints and various tissues (eg, spleen, lung) throughout the body by a hematogenous route (Hagdorn-Olsen et al., 1999a; Hagdorn-Olsen et al., 1999b).

Many factors may influence mycoplasma-associated arthritis disease expression, including maternal or herd immunity, strain virulence, management practices or biosecurity, breed, and environment (Thacker, 2006). However, it is currently unknown how each of these factors may contribute to disease pathogenesis.

**Clinical Signs**

*Mycoplasma hyorhinis* may be associated with polyserositis and polyarthritis, generally occurring in 3-to 10-week-old pigs, although occasionally it may occur in older animals (Magnusson et al., 1998; Thacker, 2006). Clinical signs typically occur 3 to 10 days after exposure and continue for approximately 10 to 14 days, depending on the severity of disease and presence of co-infections. Pigs appear unthrifty and lethargic and may demonstrate reluctance to move, lameness, and swollen joints. In addition, conjunctivitis and otitis have been associated with *M. hyorhinis* infection (Friis et al., 2002b; Kobisch et al., 1996; Morita et al., 1995; Thacker, 2006).
Mycoplasma hyosynoviae lameness generally occurs in 3- to 5-month-old pigs, appearing acutely in one or more legs (Kobisch et al., 1996; Nielsen et al., 2001a). Clinical signs that may occur in a portion of or all affected animals include lameness, stiffness, swollen joints, pain or discomfort, and reluctance to stand (Nielsen et al., 2001a; Ross et al., 1971). Clinical signs may persist for 3 to 10 days, after which lameness gradually decreases in severity. Many animals recover without treatment; however, clinical signs may persist, especially when co-infections or joints lesions such as osteochondritis dissecans are present. Swollen joints may or may not be observed in lame pigs with infectious arthritis. However, discomfort during ambulation and reluctance to stand can be frequently observed in the majority of affected pigs and suggests further evaluation.

**Animal and Sample Selection and Procedures**

Nursery, grower, or finisher pigs demonstrating lameness, swollen joints, reluctance to stand, shifting hind-limb lameness, or a painful, uncomfortable gait may be selected for sampling. Acutely affected pigs without prior antibiotic treatment are preferred for diagnostic testing. Chronically affected pigs that have demonstrated prolonged lameness, joint abscesses, recumbency, and emaciation often have other-co-infections that compromise the accuracy of diagnosis, and these animals should be avoided. Selected animals should be humanely euthanized immediately prior to sampling. Pigs that have already died should be avoided due to potential chronicity, death due to a noninfectious process, lack of a proper ante-mortem examination, or combinations of these.

Determining the correct sampling procedures to use will depend on the objectives of the investigation. Basically, two different outcomes may be pursued. The first is that of a definitive diagnosis for the clinical disease present in a particular situation. The second may be more epidemiological, that is, detection of the pathogen within a specific population to determine prevalence, incidence, or age of colonization.

Although *M. hyosynoviae* and *M. hyorhinis* may be present as commensal organisms in the upper respiratory tract of unaffected animals; confirming mycoplasma-associated arthritis
requires detection of the pathogen in affected joints and characteristic microscopic lesions in synovial tissue (Hagdor-Olsen et al., 1999b; Thacker, 2006).

Following clinical evaluation, necropsy should include an evaluation of all joints and body systems. Gross observation of serofibrinous to fibrinopurulent polyserositis and arthritis are suggestive of *M. hyorhinis* infection; however, similar lesions may be caused by infection with *Haemophilus parasuis* and *Streptococcus suis* (Kobisch et al., 1996). *Mycoplasma hyosynoviae*-affected joints tend to have large amounts of yellow or brown viscous fluid of a serofibrinous or serosanguineous nature. The synovial membrane may appear dark in color, edematous, proliferative, or hyperemic (Kobisch et al., 1996; Nielsen et al., 2001a).

Synovial fluid from elbow, stifle, hock, and shoulder should be collected using a sterile syringe and appropriately sized needle (eg, selected based on age) after removing the skin and sterilizing the region with a small propane flame to decrease bacterial contamination (Figure 3). Affected whole legs may be submitted intact to the diagnostic laboratory as an alternative to tissue samples and joint fluid.

Lung tissues should be submitted when fibrinous pleuritis is present and *M. hyorhinis* infection suspected. Pericardial, thoracic, and peritoneal fluid may also be submitted if excess effusions or polyserositis are present. Fresh and fixed heart, as well as other abdominal tissues such as liver and spleen, should be submitted in cases where polyserositis is prominent.

An alternative sampling option when insufficient synovial fluid is available may include multiple swabs aseptically collected after carefully opening the joint (Figure 4). Multiple pieces of synovium should be collected in 10% formalin and included for microscopic evaluation. The presence of characteristic microscopic lesions in synovium, in addition to pathogen detection, is required for a definitive diagnosis.

Synovial fluid from live animals can be collected from swollen joints using a sterile syringe and needle after a surgical preparation of the affected area. An advantage of this procedure is that euthanasia can be avoided; however, this technique causes discomfort to the animal and
usually requires sedation for proper sampling.

Cross-sectional samples derived from weaning- to finishing-age pigs may be used to determine the prevalence of Mycoplasma infection within a population, or prevalence may be determined longitudinally by following a specific group of animals over time. Cross-sectional studies can be used to determine the number of positive animals over time in a specific population. A disadvantage of this method is that one group of animals may not represent the prevalence of Mycoplasma infection in the entire herd. Therefore, horizontal or serial studies may be more appropriate to determine the approximate time pigs become colonized with *M. hyosynoviae* or *M. hyorhinis* or the prevalence of positive animals over time. However, either method may require evaluation of multiple groups depending on the diagnostic question and the herd situation (eg, herds that have a stable parity flow may require fewer groups to establish a pattern).

Nasal swabs and tonsil scrapings should be collected individually and not pooled for sample analysis. *Mycoplasma hyorhinis* may be detected in nasal swabs; however, tonsil scrapings are more appropriate for detecting *M. hyosynoviae*.

**Submission of Samples**

Sample submission to a diagnostic laboratory should include a complete and thorough history and clinical evaluation. A complete history should include a description and the duration of clinical signs, age and total number of affected animals, mortality, and differential diagnosis where applicable.

Synovial fluid or effusions should be transferred to polystyrene tubes prior to submission, which helps avoid leakage, cross-contamination, and damage during shipping. Synovial swabs and fluids, tissues samples, effusions, and intact joints or legs should be submitted on ice and shipped within 24 hours of collection to a diagnostic laboratory. Swabs should be in transport medium (eg, Amies medium) to prevent dehydration. Tissues submitted in 10% formalin should be properly identified and packaged appropriately to minimize the
possibility of leakage. If appropriate conditions and instruments for sampling are not available, submission of affected whole legs or a live, acutely affected animal is preferred. All samples submitted to a diagnostic laboratory should be fresh and kept cool prior to and during shipment.

**Diagnostic Tests**

Real-time polymerase chain reaction (PCR) is the most sensitive assay available to detect *Mycoplasma* species. Real-time PCR assays have been developed for both *M. hyorhinis* and *M. hyosynoviae* and may be conducted directly on clinical specimens (such as joint fluid), joint swabs, nasal swabs, or tonsil scrapings when applicable. Bacterial culture is available upon request and is necessary if further evaluation of an isolate is warranted, such as antibiotic-resistance profiles. However, bacterial cultures are time consuming and can be overgrown by other bacterial species. *Mycoplasma* species require specialized medium for growth; therefore, a specific request for Mycoplasma culture is required.

Microscopic lesions characteristic of mycoplasma infection are necessary to confirm a diagnosis of mycoplasma-associated arthritis. *Mycoplasma hyosynoviae* microscopic lesions may include hyperplasia of the synovium, moderate synovial villous hypertrophy, edema, and mononuclear cell infiltration with mild fibrosis of the capsule (Kobisch et al., 1996; Nielsen et al., 2001a). *Mycoplasma hyorhinis* typically induces a mixture of mononuclear and polymorphonuclear cells in serosal and synovial membranes, which may lead to erosion of cartilage and pannus formation in chronically affected joints (Figure 5 and 6) (Kobisch et al., 1996; Thacker, 2006).

Maternal antibodies against Mycoplasma species may be present for some time after weaning in positive herds, although the duration of maternal immunity has not been widely studied. Antibody detection in post-weaned animals may help determine the exposure status of that particular group when followed over time. Conversely, it is not uncommon to find PCR-positive animals with no detectable antibody titers (“JCGN, personal observation”). In pigs experimentally infected with *M. hyosynoviae*, antibody titers persisted for as long as 6
months post inoculation (Zimmerman et al., 1982). Mycoplasma infection demonstrated in tonsils was correlated with a significantly higher antibody response in carrier pigs. In addition, slaughter pigs with higher antibody titers were more likely to demonstrate Mycoplasma in tonsils (Nielsen et al., 2005b).

**Implications**

To diagnose mycoplasma-associated arthritis, remember:

- An accurate and complete history is critical to a definitive diagnosis of lameness in swine.
- Select untreated animals in the acute stage of lameness for submission to a veterinary diagnostic laboratory.
- Joint fluid, joint swabs, synovium in 10% formalin, or whole affected legs may be submitted to a veterinary diagnostic laboratory for lameness diagnostics.
- Ancillary tests, such as PCR or culture of joint fluid or swabs, are useful to confirm the presence of Mycoplasma species.
- A complete diagnosis of mycoplasma-associated arthritis requires the presence of microscopic lesions and identification of the organism in affected tissue.
- Communicate with the veterinary diagnostic laboratory if there are any questions.

**Acknowledgements**

The authors appreciate the cooperation of the ISU-VDL in making the database available for assessment.
References


Figure 1. Frequency of diagnosis of *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* in cases of swine lameness submitted to the Iowa State University Veterinary Diagnostic Laboratory (Ames, Iowa) between 2003 and 2010.
Figure 2. Frequency of *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* cases diagnosed by age from the total number of cases of swine lameness submitted to the Iowa State University Veterinary Diagnostic Laboratory (Ames, Iowa) between 2003 and 2010.
Figure 3. Collecting joint fluid from a 6-week-old pig demonstrating lameness prior to diagnostic testing. Photo courtesy of Dr. Philip Gauger, Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa.
Figure 4. Swabbing the intra-articular cavity for diagnostic testing. Photo courtesy of Dr. Philip Gauger, Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa.
Figure 5. Photomicrograph from a 12-week-old pig demonstrating lameness in which *Mycoplasma hyosynoviae* was detected by real-time polymerase chain reaction (PCR) in joint fluids. Chronic proliferative synovitis with mononuclear inflammation of the synovial membrane and hyperplasia and hypertrophy of synoviocytes (arrows) (H&E stain, original magnification 100x). Photo courtesy of Dr. Darin Madson, Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa.
**Figure 6.** Photomicrograph from a 6-week-old pig exhibiting swollen joints in which *Mycoplasma hyorhinis* was detected by real-time PCR in joint fluids. Fibrinosuppurative inflammation of the synovial membrane with a mixture of mononuclear and polymorphonuclear cells in the subsynovial connective tissues (*) (H&E stain, original magnification 100x). Arrows indicate hyperplasia and hypertrophy of synoviocytes. Photo courtesy of Dr. Darin Madson, Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa.
CHAPTER 2. COMPARISON OF SAMPLE TYPE AND DNA EXTRACTION PROTOCOLS FOR THE DETECTION OF *MYCOPLASMA HYOSYNOVIAE* AND *MYCOPLASMA HYORHINIS* BY REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR)

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Abstract

Three methods of DNA extraction were evaluated by comparing the rate of detection of *Mycoplasma hyosynoviae* or *M. hyorhinis* by real-time polymerase chain reaction (RT-PCR) in clinical specimens, i.e., oral fluids (n = 60), tonsil scraping fluids (n = 49), nasal swab fluids (n = 81), and serum (n = 150), collected from pigs selected from 5 commercial finisher sites. Nucleic acid extraction methods included high volume (HV) and low volume (LV) extraction using magnetic beads and a spin column-based protocol (SC). RT-PCR was performed in duplicate, and results were considered negative if cycle threshold (Ct) values were > 44. Depending on the extraction protocol, *M. hyosynoviae* DNA was detected in at least 58.3%, 9.9%, and 75.5% of pen-based oral fluids, nasal swab fluids, and tonsil scraping fluids, respectively. *M. hyorhinis* DNA was detected in at least 70% of all pen-based oral fluids and in 96.3% and 97.9% of nasal swab fluids and tonsil scraping fluids, respectively. No statistically significant differences (*P* > 0.05) in the rate of detection were observed in comparisons of DNA extraction protocols by sample type, although differences in the absolute number of positives were observed for both sample type and extraction protocol. An evaluation of extraction protocol (magnetic beads vs. spin column) across all sample types revealed more positive results associated with spin column extraction. Overall, these data suggest that tonsillar scrapings are preferable to nasal swab fluid for screening individual animals, whereas oral fluids may be useful for population surveillance.

**Key words:** *Mycoplasma hyorhinis, Mycoplasma hyosynoviae*, real-time PCR, sample types.
Introduction

Although recognized as ubiquitous in swine populations since the 1970’s, *Mycoplasma hyosynoviae* and *M. hyorhinis* have also been considered causal agents of arthritis in pigs.\(^1\),\(^2\),\(^3\),\(^4\),\(^5\),\(^6\) *Mycoplasma hyorhinis* colonizes the upper respiratory tract of pigs and is commonly associated with polyarthritis and polyserositis in weaned pigs.\(^6\),\(^7\),\(^8\) *Mycoplasma hyosynoviae* primarily colonizes tonsils of weaned pigs and may cause arthritis in 3- to 6-month-old pigs.\(^6\),\(^7\),\(^9\),\(^10\),\(^11\) Despite a long history of research, specific information is lacking regarding the epidemiology and control of the diseases associated with these microorganisms. At present, swine producers regard mycoplasma-associated arthritis as an increasing problem, impacting both animal well-being and the profitability of clinically-affected populations. Additionally, for the period 2003 to 2010, 17% of all arthritis cases diagnosed at the Iowa State University Veterinary Diagnostic Laboratory were attributed to *M. hyorhinis* and/or *M. hyosynoviae*.\(^12\)

Definitive diagnosis of mycoplasma-associated arthritis requires the isolation of the *M. hyorhinis* or *M. hyosynoviae* organism or detection of DNA in inflamed joint tissues. For purposes of surveillance or field research, these microorganisms may also be detected in tonsil homogenates, lung tissues, and nasal swabs by culture or PCR procedures.\(^13\),\(^14\),\(^15\) However, there is currently a lack of performance data on *M. hyorhinis* or *M. hyosynoviae* real-time polymerase chain reaction (RT-PCR) assays. To address this need, we compared different DNA extraction protocols and various sample matrices (pen-based oral fluids, serum, nasal swab fluids, and tonsil scraping fluids) to determine the optimal combination for the detection of these pathogens.

Materials and Methods

Experimental Design

Three methods of DNA extraction were evaluated by comparing the rate of detection of *M. hyosynoviae* or *M. hyorhinis* by RT-PCR in specimens (oral fluids, tonsil scraping fluids, nasal swab fluids, and serum) collected from 5 commercial finishing sites. Extraction
protocols differed in sample volume, the volume of lysate used in the DNA extraction, final elution volume, and the method of DNA isolation (Table 1).

**Animals and Animal Care**
The study was performed at 5 commercial finisher sites that received pigs from one sow herd. Each site had 2000 to 4000 18- to 24-week-old pigs in one or two barns. Sites were selected based on proximity to the Iowa State University Veterinary Diagnostic Laboratory and a clinical history of mycoplasma-associated arthritis in previous groups of pigs received from the same sow herd, i.e., compatible histopathology and detection of *M. hyosynoviae* and *M. hyorhinis* by RT-PCR. No vaccines against *Mycoplasma* spp. had been administered to the current group of pigs and no medications were in use at the time the experiment was conducted. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (#7-11-7181-S).

**Sample Collection**
Sampling was carried out in one day on each of the 5 sites, with the objective of collecting 12 pen-based oral fluids, blood samples from 30 pigs in the 12 pens, nasal swabs from 20 of the 30 pigs, and tonsil (oropharyngeal) scrapings from 12 of the 20 nasal swab pigs. Ultimately, a total of 340 biological specimens were collected, including 60 pen-based oral fluids, 150 serum samples, 81 nasal swabs, and 49 tonsil scrapings. Clinical specimen collection was performed seeking the optimal sample size but also considering resources availability sampling date.

Within each site, oral fluids were collected using 5/8" cotton rope attached to a partition located in the middle of each pen. The rope was cut to length so that the end was shoulder-high to the animals. Ropes were left in place for 20 to 25 minutes. To recover the oral fluid specimen, the bottom (wet end) of each rope was inserted into a large plastic boot and the oral fluid was manually extracted by squeezing the rope. The sample was decanted into a 50
ml disposable centrifuge tube for storage (Fisher Scientific Co., Pittsburgh, PA, USA). At least 30 ml of oral fluid sample was collected from each pen. Powder-free nitrile gloves were worn during the collection process and changed between each pen (Microflex Corporation, Drive Reno, NV).

Pigs for individual sampling (blood, nasal swabs, and tonsil scrapings) were selected from the pens from which oral fluid specimens were collected. Blood samples were collected using a single-use collection system (Vacuum tubes, BD, Franklin Lakes, NJ, USA). Samples were centrifuged at 2000 x g for 10 minutes, after which serum was aliquotted into 5 ml tubes and stored at -20ºC (BD Falcon™, BD Biosciences Discovery Labware, Two Oak Park, Bedford, MA, USA).

Nasal swabs were collected from each naris and placed in 5 ml polystyrene round bottom snap-cap tubes containing 2ml of 1X (PBS InvitrogenTM, Gibco, Grand Island, New York, USA). Tonsillar scrapings were collected by holding the mouth open with an oral speculum and scraping the surface of the tonsil using a sterilized, blunt stainless steel spoon with an elongated handle after restraining the animals with a nose snare.16 Tonsil scraping fluids were placed in 5 ml tubes containing 3 ml of sterile PBS 1X (previously tested negative for RT-PCR for both pathogens) and stored at -20 ºC until tested (BD Falcon™, BD Biosciences Discovery Labware, Two Oak Park, Bedford, MA, USA; InvitrogenTM, Gibco, Grand Island, New York, USA).

**Nucleic Acid Extraction and Real-Time Polymerase Chain Reaction (RT-PCR)**

Three methods of nucleic acid extraction were evaluated: (1) High volume nucleic acid extraction using magnetic beads (HV) (MagMAX™ Viral RNA Isolation Kit, Applied Biosystems, Foster City, CA, USA); (2) Low volume nucleic acid extraction using magnetic beads (LV) (MagMAX™ Viral RNA Isolation Kit, Applied Biosystems, Foster City, CA, USA); and (3) spin column nucleic acid extraction (High Pure PCR Template Preparation Kit, Roche, Indianapolis, IN, USA).
**High volume (HV) and low volume (LV) nucleic acid extraction using magnetic beads**

HV and LV extraction protocols were based on a commercial kit and the samples were processed according to the manufacturer’s instructions. The HV protocol used 300 μl of uncentrifuged pen-based oral fluids samples; the LV protocol used 50 μl of nasal swab fluid, tonsil scraping fluid, or serum. After loading with samples, plates were placed onto a semi-automated nucleic acid purification system along with two plates each of washing solution 1 and 2, and a plate of elution buffer solution, as described by the manufacturer (KingFisher® 96 magnetic particle processor, Thermo Fisher Scientific Inc., Waltham, MA, USA). The HV protocol (oral fluid specimens) used the software program AM1836_DW_HV_v3; the LV protocol (nasal swab fluid, tonsil scraping fluid, and serum) used the program 1836_DW_50_v3 (Applied Biosystems, Foster City, CA, USA). Thereafter, the elution plate was sealed and frozen at -20ºC until it was assayed by RT-PCR (AluminaSeal™, Diversified Biotech, Dedham, MA, USA).

**Spin-column based nucleic acid extraction protocol (SC)**

Protocol SC was based on a commercial kit and was performed as directed by the manufacturer (High Pure PCR Template Preparation Kit, Roche, Indianapolis, IN, USA). A volume of 200 μl of uncentrifuged oral fluid, tonsil scraping fluid, nasal swab fluid, and serum was used for the reaction. To elute the nucleic acid, 100 μl of pre-warmed (70ºC for 5 minutes) elution buffer was used. Thereafter, all samples were then frozen at -20ºC until assayed by RT-PCR.

**Real-time PCRs for *M. hyorhinis* and *M. hyosynoviae***

For this reaction, amplified products of *M. hyosynoviae* and *M. hyorhinis* were 397 and 161bp in length, respectively. The 16s rRNA was the target sequence for both pathogens. In brief, 22.5 μl of a solution composed of 12.5 μl of QuantiTect® SYBR® Green master mix (Qiagen® Inc., Valencia, CA, USA), one μl of 10 µM stock solution of reverse and forward primers for either *M. hyosynoviae* or *M. hyorhinis*, and 8 μl of nuclease-free water (Qiagen® Inc., Valencia, CA, USA) was pipetted into each well of a 96 well plate (Fast® PCR plates, Applied Biosystems, Foster City, CA, USA). *Mycoplasma hyosynoviae* forward and reverse primers (Integrated DNA Technologies, Inc., San Diego, California) were: 5’- CAG TTG AGG AAA TGC AAC TGA AC -3’ and 5’- CGT CAG TGA TTG GCC ACC G -3’, respectively. *Mycoplasma hyorhinis*
forward and reverse primers were: 5’-TGT TGA ACG GGA TGT AGC AA -3’ and 5’-TGA AGC TGT GAA GCT CCT TTC -3’, respectively. DNA-extracted material (2.5 μl) was then added to each well.

RT-PCR was then performed using a 96-well thermal cycler using the following cycling conditions: 1 cycle at 95°C for 10 minutes, 45 cycles at 95°C for 15 seconds, 59°C for 30 sec (M. hyorhinis) or 63°C for 30 sec (M. hyosynoviae), followed by a melting curve of 72°C for 30 sec, 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 seconds (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Quality controls included on each plate consisted of one well of previously DNA-extracted from nuclease-free water (negative control) and pure cultures of either M. hyorhinis or M. hyosynoviae (positive control). Analysis of amplification curves was performed using the manufacturer's software (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Auto baseline was used to determine fluorescence baselines, and the threshold was manually set at 0.04. For either organism, a sample was considered positive if the Ct value was ≤ 44 and the melting temperature (Tm) was 75.7°C ± 0.5°C or 81.4°C ± 0.5°C, for M. hyorhinis and M. hyosynoviae, respectively. RT-PCR reactions were done in duplicate. In the case of discordant results, i.e., one positive and one negative, the sample was re-tested to achieve a consensus.

**Statistical Methods**

Statistical analysis was performed using commercial software SAS® Version 9.2 (SAS® Version 9.2, SAS Institute Inc., Cary, NC, USA). Differences in the detection of M. hyosynoviae and M. hyorhinis by DNA extraction protocol (high volume, low volume, spin column) were evaluated by sample type (OF, NS, TS) using Cochran’s Q test and McNemar's chi-square test. Cochran’s Q test was performed for each pathogen to detect differences in the detection rate among the four diagnostic tests. Post-hoc pairwise comparisons between the binary diagnostic test results were done using McNemar's chi-square test.
Results

Overall, *M. hyorhinis* and *M. hyosynoviae* DNA were detected in all clinical specimens except for serum samples. Depending on the extraction protocol, *M. hyosynoviae* DNA was detected in at least 58.3%, 9.9%, and 75.5% of pen-based oral fluids, nasal swab fluids, and tonsil scraping fluids, respectively (Table 2). *Mycoplasma hyorhinis* DNA was detected in at least 70% of all pen-based oral fluids and in 96.3% and 97.9% of nasal swab fluids and tonsil scraping fluids, respectively (Table 2). No statistically significant differences (*P* > 0.05) in the rate of detection were observed in comparisons of DNA extraction protocols by sample type for either *M. hyosynoviae* or *M. hyorhinis*, although differences in the absolute number of positives were observed for both sample type and extraction protocol (Table 2). A comparison of tonsil vs. nasal swab nucleic acid detection rates revealed that a statistically higher proportion of tonsil scraping fluids were positive for *M. hyosynoviae* irrespective of the DNA extraction method (Table 3). In contrast, *M. hyorhinis* DNA was detected in 97.9% of both nasal swab and tonsil scraping fluids, regardless of the DNA extraction protocol utilized. An evaluation of extraction protocol (magnetic beads vs. spin column) across all sample types revealed a numeric difference in the results, with more positive results associated with spin column extraction (Table 4). This difference was statistically significant for *M. hyosynoviae* (*P* = 0.0209), but not for *M. hyorhinis* (*P* = 0.1892).

Discussion

*Mycoplasma hyosynoviae* and *M. hyorhinis* can be isolated from the tonsil, nasal cavity, and synovial fluids of both experimentally-challenged and naturally-exposed animals using standard culture procedures. However, laboratory culture of these slow-growing organisms from clinical specimens is complicated by overgrowth of the bacteria invariably present in these samples. While the use of antibiotics may improve isolation by inhibiting the growth of competing microorganisms, recovery of mycoplasma species in the
diagnostic laboratory remains challenging. As an alternative to culture, RT-PCR or enrichment followed by RT-PCR, promises a significant improvement in diagnostic performance, but this potential that can only be achieved through comparative studies that identify the optimum protocols and specimens.\textsuperscript{22,23} Thus, the objective of the present study was to compare nucleic acid extraction protocols and specific sample matrices, i.e., serum, tonsil scraping fluids, nasal swab fluids, and pen-based oral fluids, in order to identify the optimal diagnostic protocol for the detection of these pathogens.

\textit{Mycoplasma hyosynoviae} has been cultured from the blood of infected pigs\textsuperscript{9,20}, but all serum samples were negative for both \textit{M. hyosynoviae} and \textit{M. hyorhinis} in this study potentially indicating the true absence of bacteremia due to lack of clinical signs in the population at that point in time or a low sensitivity provided by this sample type. In contrast, both \textit{M. hyosynoviae} and \textit{M. hyorhinis} DNA were detected in tonsil scraping fluids, nasal swab fluids, and pen-based oral fluids. \textit{Mycoplasma hyosynoviae} was more commonly detected in tonsil scraping vs. nasal swab fluids (Table 3), whereas \textit{M. hyorhinis} was detected in both nasal swab and tonsil scraping fluids. These results may reflect either the stage of infection or possible differences in tissue tropism (tonsil vs. nasal cavity); however, this hypothesis would have to be appropriately investigated under experimental condition. While previous research has demonstrated that a variety of pathogens may be detected in porcine oral fluids, e.g., influenza virus, \textit{Actinobacillus pleuropneumoniae}, \textit{Erysipelothrix rhusiopathiae}, porcine respiratory and reproductive syndrome virus (PRRSV), \textit{Porcine circovirus} type 2, and others\textsuperscript{24-30}, this study confirmed that \textit{M. hyosynoviae} and \textit{M. hyorhinis} may also be detected by RT-PCR in pen-based oral fluids samples.

Comparisons have shown significant differences among nucleic acid extraction protocols in the rate of PRRSV detection in oral fluids.\textsuperscript{31} No statistically significant differences in detection rates were detected when comparing DNA extraction protocols within each of the three sample types, but a higher proportion of positive samples were uniformly observed with the spin column DNA extraction protocol for all sample types (Table 2). Collapsing the data and comparing the detection rate across all sample types revealed a statistically significant difference in the proportion of \textit{M. hyosynoviae}-positive samples detected with spin column
vs. magnetic bead extraction. No significant difference existed for *M. hyorhinis*-positive samples.

Overall, this study provided guidelines for the detection of *M. hyosynoviae* and *M. hyorhinis* in clinical specimens submitted to the diagnostic laboratory. More specifically, these data suggest that tonsil scraping fluid is preferable to nasal swab fluid for screening individual animals, and oral fluids may be useful for population surveillance. Furthermore, the data suggest that spin column DNA extraction may provide better diagnostic sensitivity, but it should be recognized that molecular diagnostics are in a period of rapid evolution. Therefore, future comparative studies will be required.
Implications

1. *Mycoplasma hyosynoviae* and *M. hyorhinis* could be detected in nasal swabs, oral fluids and tonsillar scrapings regardless of the DNA extraction protocol utilized.

2. Tonsillar scrapings are preferable to nasal swab fluid for screening individual animals for either pathogen, although *M. hyorhinis* was successfully detected in nasal swabs.

3. The presence of these organisms in oral fluids opens a new opportunity for understanding the dynamic of infection in endemic herds; although, improvements may be necessary to maximize the assay performance.

4. Detection of these pathogens in pig populations should be combined with clinical data in future studies to better understand the usefulness of these new diagnostics tools and routinely used sample methods.

Acknowledgements

We would like to thank Brent Pepin, Dr. Todd Wolf, and Kent Naughton for their help in sample collection.

Declaration of Conflicting Interests

The authors declare no conflicting interests with respect to their authorship or the publication of this article.

Funding

The study was supported by funding through the Iowa State University, Veterinary Diagnostic Laboratory.
References


10. Hagedorn-Olsen T, Nielsen NC, Friis NF. Induction of arthritis with *Mycoplasma hyosynoviae* in pigs: Clinical response and re-isolation of the organism from body fluids


<table>
<thead>
<tr>
<th>Extraction Protocol</th>
<th>Sample type*</th>
<th>Sample volume</th>
<th>Lysis solution</th>
<th>Elution buffer</th>
<th>DNA capture</th>
</tr>
</thead>
<tbody>
<tr>
<td>High volume</td>
<td>OF</td>
<td>300 μl</td>
<td>450 μl</td>
<td>90 μl</td>
<td>Magnetic bead</td>
</tr>
<tr>
<td>Low volume</td>
<td>S, NS, TS</td>
<td>50 μl</td>
<td>130 μl</td>
<td>50 μl</td>
<td>Magnetic bead</td>
</tr>
<tr>
<td>Spin column</td>
<td>OF, S, NS, TS</td>
<td>200 μl</td>
<td>200 μl</td>
<td>100 μl</td>
<td>Silica gel</td>
</tr>
</tbody>
</table>

* OF (oral fluid), S (serum), NS (nasal swab fluid), and TS (tonsil scraping fluid) collected from pigs in commercial production systems
Table 2. Summary of RT-PCR-positives (%) by sample type, and extraction protocol

<table>
<thead>
<tr>
<th>Sample type*</th>
<th>Extraction protocol†</th>
<th>Diagnostic agreement</th>
<th>P value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>High volume</td>
<td>Low volume</td>
<td>Spin column</td>
<td></td>
</tr>
<tr>
<td><em>M. hyosynoviae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OF (n = 60)</td>
<td>35 (58.3%)</td>
<td>Not applicable</td>
<td>39 (65.0%)</td>
</tr>
<tr>
<td>NS (n = 81)</td>
<td>Not applicable</td>
<td>8 (9.9%)</td>
<td>12 (14.8%)</td>
</tr>
<tr>
<td>TS (n = 49)</td>
<td>Not applicable</td>
<td>37 (75.5%)</td>
<td>42 (85.7%)</td>
</tr>
<tr>
<td><em>M. hyorhinis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OF (n = 60)</td>
<td>42 (70.0%)</td>
<td>Not applicable</td>
<td>49 (81.7%)</td>
</tr>
<tr>
<td>NS (n = 81)</td>
<td>Not applicable</td>
<td>78 (96.3%)</td>
<td>79 (96.3%)</td>
</tr>
<tr>
<td>TS (n = 49)</td>
<td>Not applicable</td>
<td>48 (97.9%)</td>
<td>48 (97.9%)</td>
</tr>
</tbody>
</table>

* Oral fluid (OF), nasal swab fluid (NS), and tonsil scraping fluid (TS)
† Number and proportion of positive samples
‡ Comparisons between extraction protocols by sample type (McNemar’s chi-square test)
Table 3. Pairwise comparison* of the detection rate of *Mycoplasma hyosynoviae* nucleic acid by sample type and DNA extraction protocol

<table>
<thead>
<tr>
<th>Sample type and DNA extraction protocol (number of RT-PCR positive results)</th>
<th>TSMB (n = 37†)</th>
<th>NSMB (n = 6)</th>
<th>TSSC (n = 42)</th>
<th>NSSC (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSMB - Tonsil scraping fluid extracted using low volume-magnetic beads protocol (n = 37)</td>
<td>NA</td>
<td>( P &lt; 0.0001 )</td>
<td>( P = 0.0956 )</td>
<td>( P &lt; 0.0001 )</td>
</tr>
<tr>
<td>NSMB - Nasal swab fluid extracted using low volume-magnetic beads protocol (n = 6)</td>
<td>( P &lt; 0.0001 )</td>
<td>NA</td>
<td>( P &lt; 0.0001 )</td>
<td>( P = 0.5637 )</td>
</tr>
<tr>
<td>TSSC - Tonsil scraping fluid extracted using spin column protocol (n = 42)</td>
<td>( P = 0.0956 )</td>
<td>( P &lt; 0.0001 )</td>
<td>NA</td>
<td>( P &lt; 0.0001 )</td>
</tr>
<tr>
<td>NSSC - Nasal swab fluid extracted using spin column protocol (n = 7)</td>
<td>( P &lt; 0.0001 )</td>
<td>( P = 0.5637 )</td>
<td>( P &lt; 0.0001 )</td>
<td>NA</td>
</tr>
</tbody>
</table>

* \( P \) values calculated using McNemar’s chi-square test.
† Number of RT-PCR positive samples among 49 samples tested.
Table 4. Pairwise comparison of the detection rate of *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* by DNA extraction protocol across all sample types*

<table>
<thead>
<tr>
<th>Mycoplasma spp.</th>
<th>Extraction protocol</th>
<th>P value‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Magnetic beads†</td>
<td>Spin column</td>
</tr>
<tr>
<td><em>M. hyosynoviae</em></td>
<td>80 (42.1%)§</td>
<td>93 (48.9%)</td>
</tr>
<tr>
<td><em>M. hyorhinis</em></td>
<td>168 (88.4%)</td>
<td>175 (92.1%)</td>
</tr>
</tbody>
</table>

* Oral fluid (n = 60), nasal swab fluid (n = 81), and tonsil scraping fluid (n = 49), as given in Table 1.

† Includes both high- and low-volume magnetic bead extraction protocols

‡ Comparisons between extraction protocols by pathogen (McNemar's chi-square test)

§ Number and proportion of positive samples
CHAPTER 3. LONGITUDINAL INVESTIGATION OF *MYCOPLASMA HYOSYNOVIAE* AND *MYCOPLASMA HYORHINIS* INFECTIONS IN A WEAN-TO-FINISH POPULATION

Submitted to the *Journal of Swine Health and Production*

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Abstract

Given the limited epidemiological information available on *Mycoplasma hyosynoviae* and *M. hyorhinis* infections, the objective of this longitudinal study was to describe the circulation of these pathogens in a commercial wean-to-finish swine population. Pigs in a wean-to-finish barn housing 2,420 animals (220 pigs per pen) were monitored for *M. hyorhinis* and *M. hyosynoviae* infections using serum and pen-based oral fluid samples collected at placement and each ~30 days thereafter. Diagnostic results were evaluated as the proportion of real-time polymerase chain reaction (RT-PCR) positive oral fluid samples and changes in mean serum antibody ELISA optical density (OD) response over time. *M. hyorhinis* RT-PCR-positive oral fluid specimens were detected at 28 days post placement (DPP), whereas *M. hyosynoviae* was not detected until 88 DPP. Thereafter, all pen-based oral fluid samples were RT-PCR positive for both organisms through 148 DPP. Mean serum antibody ELISA OD responses reflected RT-PCR results for both organisms. *M. hyorhinis* OD values declined significantly (*P* < 0.05) after 0 DPP and then increased significantly at each sampling point thereafter, reaching a plateau at 88 DPP. The pattern was similar for *M. hyosynoviae* OD values, although the timing was somewhat delayed compared to *M. hyorhinis*. Overall, distinctly different patterns of shedding were demonstrated for each mycoplasma in this population, but the patterns of circulation were consistent with previous reports. The combination of specimen types and diagnostic assays could be used to in the future to explore the dynamics of mycoplasmal infections in endemically-infected swine populations.

**Key-words:** Swine, *Mycoplasma hyorhinis, Mycoplasma hyosynoviae*, ELISA, real time-PCR
Introduction

*Mycoplasma hyosynoviae* and *M. hyorhinis* infections are common in swine populations, with pigs becoming infected by contact with sows or older pigs in the system (Hagerdon-Olsen et al., 1999; Ross et al., 1973; Thacker and Minion, 2012). Pigs are believed to become infected with *M. hyosynoviae* in the early growing phase (8 to 12 weeks), while *M. hyorhinis* is more likely to be found in pigs immediately after weaning (Clavijo et al., 2012, Kobisch et al., 1996, Nielsen et al., 2001, Thacker and Minion, 2012). Primary replication occurs in the upper respiratory tract, followed by dissemination to other tissues (Thacker and Minion, 2012). Given the limited epidemiological information available, the objective of this longitudinal study was to describe the circulation of these pathogens in a commercial wean-to-finish swine population.

Materials and Methods

Experimental Design

Pigs in a wean-to-finish barn housing 2,420 animals (220 pigs per pen) were monitored for 148 days post placement (DPP) for evidence of exposure to *M. hyorhinis* and *M. hyosynoviae*. Samples collected at placement and approximately every 30 days thereafter were tested using a Tween 20 antibody ELISA (serum) and real-time polymerase chain reaction (RT-PCR) (pen-based oral fluids) for evidence of *M. hyorhinis* and *M. hyosynoviae* infections. Results were evaluated as changes in antibody response (optical density) and the proportion of RT-PCR positive samples over time.

Animals and Animal Care

The study was performed in one wean-to-finish barn with pigs placed at approximately 3 weeks of age. The barn housed a total of 2420 animals in 11 pens, i.e., 220 animals per pen. The production site was selected based on its clinical history. That is, both the sow herd from which the pigs were sourced and previous groups of pigs sourced from the sow herd
had a history of mycoplasma-associated arthritis. No vaccines or specific treatments against either *M. hyosynoviae* or *M. hyorhinis* were used during the observation period and animal care and management protocols were consistent with previous batches. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee.

**Sample Collection**
At placement, 5 pens evenly distributed throughout the barn were selected for longitudinal sampling and 6 pigs from each pen were ear-tagged for identification. Thereafter, pen-based oral fluids and blood samples were collected by caretakers and the herd veterinarian at 6 time points from placement through 150 DPP, i.e., 0, 28, 58, 88, 118, and 148 DPP.

Blood samples were collected using a single-use collection system (BD Vacutainer®, BD Diagnostics, Franklin Lakes, NJ, USA), centrifuged at 2000 x g for 10 minutes, and the harvested serum stored at -20°C until tested.

Pen-based oral fluids were collected using cotton rope (5/8 inches or 1.59 cm) attached to a partition located in the middle of each pen, as described elsewhere (Prickett et al., 2008). The rope was cut such that the end was at shoulder level to the pigs in the pen. Ropes were left in place for 20 to 25 minutes, during which time the pigs chewed the rope, depositing oral fluids in the process. To recover the oral fluid, the bottom (wet) portion of each rope (10 to 12 inches) was inserted into a disposable plastic boot and the oral fluids extracted by manually squeezing the rope. The fluid recovered in the bag (~30 ml) was decanted into tubes and stored at -20°C until tested. Nitrile gloves (Microflex Corporation, Drive Reno, NV) were used throughout the collection process and were changed between each pen.

**Nucleic Acid Extraction and Real-Time Polymerase Chain Reaction (RT-PCR)**
*High volume nucleic acid extraction using magnetic beads*  The extraction protocol was based on a commercial kit (MagMAX™ Viral RNA Isolation Kit, Applied Biosystems,
Foster City, CA, USA) and the samples were processed according to the manufacturer’s instructions. In brief, after loading the plates with 300 μl of unprocessed pen-based oral fluids samples, plates were placed onto a semi-automated nucleic acid purification system (KingFisher® 96 magnetic particle processor, Thermo Fisher Scientific Inc., Waltham, MA, USA) along with 2 plates of washing solution 1, 2 plates of washing solution 2, and one plate of elution buffer solution, as described by the manufacturer. The software program used was AM1836_DW_HV_v3 (Applied Biosystems, Foster City, CA, USA). Thereafter, the elution plate was sealed (AluminaSeal™, Diversified Biotech, Dedham, MA, USA) and frozen at -20ºC until it was assayed by RT-PCR.

Real-time PCRs for M. hyorhinis and M. hyosynoviae  In brief, 22.5 μl of a solution composed of 12.5 μl of QuantiTect® SYBR® Green master mix (Qiagen® Inc., Valencia, CA, USA), one μl of 10 μM stock solution of reverse and forward primers (Integrated DNA Technologies, Inc., San Diego, California) for either M. hyosynoviae or M. hyorhinis, and 8 μl of nuclease-free water (Qiagen® Inc., Valencia, CA, USA) was pipetted into each well of a 96-well plate (Fast® PCR plates, Applied Biosystems, Foster City, CA, USA). M. hyosynoviae forward and reverse primers were: 5’- CAG TTG AGG AAA TGC AAC TGA AC -3’ and 5’- CGT CAG TGA TTG GCC ACC G -3’, respectively. M. hyorhinis forward and reverse primers were: 5’- TGT TGA ACG GGA TGT AGC AA -3’ and 5’- TGA AGC TGT GAA GCT CCT TTC -3’, respectively. DNA-extracted material (2.5 μl) was then added to each well. For this reaction, amplified products of M. hyosynoviae and M. hyorhinis were 397 and 161bp in length, respectively. RT-PCR assays targeted the 16S rRNA sequence of either pathogen.

RT-PCR was performed using a 96-well thermal cycler (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: one cycle at 95ºC for 10 minutes, 45 cycles at 95ºC for 15 seconds, 59ºC for 30 sec (M. hyorhinis) or 63ºC for 30 sec (M. hyosynoviae), followed by a melting curve of 72ºC for 30 sec, 95ºC for 15 sec, 60ºC for 1 min, and 95ºC for 15 seconds. Quality controls included on each plate consisted of one well of previously DNA-extracted nuclease-free water (negative control) and a pure culture of either M. hyorhinis or M. hyosynoviae (positive control).
Analysis of amplification curves was performed using the manufacturer's software (7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA). Auto baseline was used to determine fluorescence baselines, and the threshold was manually set at 0.04. For either organism, a sample was considered positive if the Ct value was ≤ 44 and the melting temperature (Tm) was 75.7°C ± 0.5°C or 81.4°C ± 0.5°C, for *M. hyorhinis* and *M. hyosynoviae*, respectively. RT-PCR reactions were done in duplicate. In the case of discordant results, i.e., one positive and one negative, the sample was re-tested to achieve a consensus.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Serum samples were tested in duplicate for the presence of antibodies against *M. hyosynoviae* and *M. hyorhinis* using an adaptation of a Tween 20 assay previously described for *M. hyopneumoniae* (Bereiter et al., 1990). In brief, the antigens for the ELISAs were extracted from whole-cell cultures of *M. hyorhinis* and *M. hyosynoviae* using a Tween 20 detergent reagent. Plates were individually prepared for each organism. Wells of 96-well plates (Nunc Immulon 2 HB, Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated with 100 μl of 10 μg per ml of each antigen in carbonate-bicarbonate buffer (pH 9.6). Thereafter, plates were incubated overnight at room temperature and then stored at ~80°C.

To perform the assay, plates were thawed and the wells hand-washed 3 times with 200 μl of wash solution composed of 0.85% NaCl (Fisher Scientific Co., Pittsburgh, PA, USA) and 0.05% Tween® 20 (Sigma-Aldrich® Co. LLC, Saint Louis, MO, USA). Thereafter, 100 μl of serum diluted 1:50 in Tris buffered saline (Tris HCl (THAM) H₂NC(CH₂OH)₃, Fisher Scientific Co., Pittsburgh, PA, USA) was added to each well, the plates incubated at 37°C for 30 minutes, and then the plates were washed 3 times with wash solution. One positive and one negative control, i.e., serum from animals of known status, were run on each plate as quality controls. To detect reactions, 100 μl of a 1:10,000 dilution of peroxidase-conjugated rabbit anti-pig IgG (ICN Biomedicals, Irvine, CA, USA) was dispensed into each well, after which the plates were incubated for 30 minutes at 37°C and then washed 3 times. TMB
substrate-chromogen (100 μl) (TMB One-Step Substrate System, Dako North America Inc., Carpinteria, CA, USA) was added to each well, plates were incubated for an additional 8 minutes at 37°C, and then 100 μl of stop solution (PRV gI Antibody Test, Idexx Laboratories, Westbrook, ME, USA) was dispensed into each well. Plates were read at 405 nm (Model AL310, Bio-Tek instruments, Inc., Winooski, VT, USA) and the results given as optical density (OD). For each plate, sample results were adjusted by subtracting the OD of the negative control.

**Statistical Methods**

Statistical analysis of the data was performed using the MedCalc® Version 12.2.1.0 (Broekstraat 52, 9030 Mariakerke, Belgium). Pen-based oral fluid RT-PCR data and individual pig serum ELISA OD results were grouped by (DPP). Initially, summary statistics were calculated for all groups to assess the overall quality of the data including normality. Repeated measure analysis of variance (ANOVA) was then used to evaluate serum OD values for quantitative differences in response among groups over time. Values of $P < 0.05$ were defined as statistically significant.

**Results**

Testing of pen-based oral fluids by RT-PCR revealed distinctly different patterns of circulation (*M. hyorhinis* vs. *M. hyosynoviae*) in the monitored population. *M. hyorhinis* RT-PCR-positive oral fluid specimens were detected in samples on day 28 DPP, whereas *M. hyosynoviae* was not detected until 88 DPP (Figures 1 and 2). Thereafter, all tested samples were positive for both organisms through 148 DPP. Mean serum ELISA OD values reflected RT-PCR results for both organisms. As shown in Figure 1, *M. hyorhinis* OD values declined significantly ($P < 0.05$) after 0 DPP and then increased significantly at each sampling point thereafter, reaching a plateau at 88 DPP. As shown in Figure 2, the pattern was similar for *M. hyosynoviae*, although the timing was somewhat delayed compared to *M. hyorhinis*. 
Discussion

*Mycoplasma*-associated arthritis is increasingly recognized for its impact on both animal well-being and the productivity of clinically-affected populations. As an example of this trend, 17% of all arthritis cases diagnosed at the Iowa State University Veterinary Diagnostic Laboratory from 2003 to 2010 were attributed to *M. hyorhinis* and/or *M. hyosynoviae* (Gomes Neto et al., 2012). Even so, interpretation of the apparent changes in the presentation and/or clinical importance of these pathogens is compromised by a lack of information describing their epidemiology in the field. Different patterns of *M. hyosynoviae* infection have been reported in naturally-exposed populations. In the course of investigating three herds, Hagerdon-Olsen et al. (1999) found that infection was limited to adult animals in one herd, but occurred in all stages of production in two herds; both with and without antibody production and in the presence or absence of clinical arthritis. There is even less descriptive epidemiology available on *M. hyorhinis* infection. Essentially, our current knowledge is based on data from Veterinary Diagnostic Laboratories or prevalence studies based on specimens collected at slaughter (Falk et al., 1991, Gomes Neto et al., 2012, Makhanon et al., 2011, Palzer et al., 2008, Schulman et al., 1970).

The objective of this longitudinal study was to describe the circulation of these pathogens in a commercial wean-to-finish swine population via collection and testing of both serum and pen-based oral fluid specimens. Serum antibodies were used to indirectly follow the course of the infection and monitor the development of humoral immunity in the herd. The use of oral fluid samples was justified by the fact that both *M. hyosynoviae* and *M. hyorhinis* are commonly detected in tonsils of infected animals (Clavijo et al., 2012, Hagedorn-Olsen et al., 1999, Kobisch et al., 1996, Ross et al., 1973, Thacker and Minion, 2012). This approach was also supported by the work that has been done with other pathogens, e.g., *Haemophilus parasuis, Streptococcus suis, Actinobacillus pleuropneumoniae, Erysipelothrix rhusiopathiae*, swine influenza, porcine circovirus type 2, porcine respiratory and reproductive syndrome virus, and others (Bender et al., 2010, Costa et al., 2012, Prickett et al., 2010, Ramirez et al., 2012).
Although the detection of serum antibody against *M. hyosynoviae* or *M. hyorhinis* has not been proven to correlate with the disease status of individual animals, it may be useful for evaluating exposure and/or vaccine compliance at the population level (Hagedorn-Olsen et al., 1999, Nielsen et al., 2005). A variety of assays have been used to detect antibodies against *M. hyosynoviae* and *M. hyorhinis*, e.g., the metabolic inhibition test, indirect hemagglutination, complement fixation, and latex-agglutination (Freeman et al., 1984, Ross et al., 1972, Zimmerman et al., 1977, Zimmerman et al., 1982). In addition, a Tween 20 ELISA assay has long been used for the detection of antibodies against *M. hyopneumoniae*; thus, suggesting its possible application for other swine mycoplasmas (Bereiter et al., 1990).

In this study, a decline in the mean *M. hyorhinis* and *M. hyosynoviae* OD values post weaning was suggestive of decaying maternal immunity; with the subsequent increase in serum antibody indicative of extensive spreading and an adaptive immune response (Figures 1 and 2). This interpretation of the serological response was supported by RT-PCR testing of pen-based oral fluids. Although no RT-PCR positive samples were detected at placement, the majority of samples were positive from 28 DPP to the end of the observation period at 148 DPP (Figure 1). In contrast, oral fluid specimens were negative for *M. hyosynoviae* until 88 DPP, after which most samples were positive through 148 DPP (Figure 2). Within this population, distinctly different patterns of shedding were demonstrated for each microorganism using RT-PCR testing of pen-based oral fluid samples, but the patterns of circulation were consistent with previous reports (Clavijo et al., 2012, Hagerdon-Olsen et al., 1999).

Despite evidence of extensive transmission and infection, no overt clinical effects attributable to either *M. hyorhinis* or *M. hyosynoviae* were observed. These observations leave large questions concerning the role of maternal and protective immunity in the control of the infection unanswered, but demonstrate that further field studies could be conducted using this approach. Regardless, the current study demonstrates that a combination of multiple samples types and diagnostic assays may provide insight into the dynamics of mycoplasmal infections in endemically-infected swine populations.
Implications

- Real-time PCRs for *M. hyorhinis* and *M. hyosynoviae* can be used with pen-based oral fluids to understand the circulation of these pathogens.

- The use of either *M. hyosynoviae* or *M. hyorhinis* Tween 20 ELISA assays can contribute to an understanding of the role of maternal and/or adaptive humoral immunity in infected populations.

Acknowledgements

We would like to thank the farm caretakers and Dr. Todd Wolf for the help with the sample collection.

Funding

The laboratory testing was supported by the Iowa State University, Veterinary Diagnostic Laboratory.

References


10. Makhanon M, Tummaruk P, Thongkamkoon P, Thanawongnuwech R, Prapasarakul N. Comparison of detection procedures of *Mycoplasma hyopneumoniae, Mycoplasma hyosynoviae,* and *Mycoplasma hyorhinis* in lungs, tonsils, and synovial fluid of


19. Schulman A, Estola T, Garry-Andersson AS. On the occurrence of *Mycoplasma*
hyorhinis in the respiratory organs of pigs, with special reference to enzootic pneumonia. 


**Figure 1.** ELISA antibody response (left y-axis-gray line) against *M. hyorhinis* in pigs through 148 DPP. Values are reported as group mean O.D. ± S.E.M. (*asterisks represent time-points with statistically significant difference in mean OD values). Proportion of RT-PCR positive pen-based oral fluids (right y-axis-blue bars). a,b,c,d Mean OD values without a common superscript differ ($P < 0.05$).
**Figure 2.** ELISA antibody response (left y-axis-red line) against *M. hyosynoviae* in pigs through 148 DPP. Values are reported as group mean O.D. ± S.E.M. (* asterisks represent time-points with statistically significant difference in mean OD values). Proportion of RT-PCR positive pen-based oral fluids (right y-axis-gray bars). Mean OD values without a common superscript differ (*P* <0.05).
GENERAL CONCLUSIONS

Advances in swine medicine have unquestionably changed the views and attitudes of producers, practitioners, and researchers on how to produce meat in a socially responsible, welfare friendly, and economically sustainable manner. Surveillance and control of emerging and re-emerging diseases by national and international institutions is part of this equation. In this context, mycoplasma-associated arthritis may be regarded as a re-emergent issue with important economic and welfare implications. That is, animals affected by mycoplasma-associated arthritis require treatment or humane euthanasia. Preferable to either of these courses is prevention of mycoplasmal diseases through prophylactic approaches. Therefore, the objective of this thesis was to explore new diagnostic approaches to be used to better understand the diagnostics and epidemiology of *Mycoplasma hyosynoviae* and *M. hyorhinis*.

Molecular diagnostic techniques have been rapidly implemented in veterinary diagnostic laboratories. However, these tools cannot be used effectively until they have been validated for each pathogen and diagnostic specimen. A major purpose of the research described within this thesis was to investigate the applicability of real-time PCR to the detection of *M. hyosynoviae* and *M. hyorhinis* in specimen types routinely submitted to veterinary diagnostic laboratories. Interestingly, both *M. hyosynoviae* and *M. hyorhinis* were detected in pen-based oral fluids samples. Likewise, tonsil scraping samples were also highly effective at detecting these pathogens in individual pigs. Subsequently, these diagnostic tools - in tandem with serum ELISAs - were used to monitor the circulation of these pathogens in a wean-to-finish population over time. In this particular population, distinct patterns of infection were demonstrated for each microorganism.

Overall, this work demonstrated the practical applications of these new technologies to field and diagnostic situations. However, we are also left with challenging questions concerning the epidemiology, pathogenesis, and diagnosis of *M. hyosynoviae* and *M. hyorhinis*. For example, many herds infected with *M. hyosynoviae* or *M. hyorhinis* never present evidence of clinical disease, despite the chronic presence of these mycoplasmas in pen-based oral fluids,
nasal swabs fluids, or tonsil scrapings. Why are some pigs clinically affected by these infections and the majority are not? At this juncture, we have little understanding of the role of mycoplasmal genetic evolution or virulence factors in the expression of disease. Likewise, pig genetics have resulted in a highly productive phenotype, but whether host tolerance or susceptibility to these agents has been affected in this process is unknown. For the immediate future, there is a clear need to commit resources toward elucidating the clinical and economic impact of mycoplasma-associated arthritis in the contemporary swine industry. For the long term solution, the core question revolves around the mechanisms of pathogenesis and the triggers of clinical disease. The good news is that, in spite of these lingering unknowns, we are better equipped than ever before to diagnose *M. hyosynoviae* and *M. hyorhinis* in the diagnostic laboratory and conduct studies in the field.
ACKNOWLEDGEMENTS

I would like to thank the faculty and staff from the departments of Veterinary Diagnostic and Production Animal Medicine, Veterinary Microbiology and Preventive Medicine, and the Iowa State University Veterinary Diagnostic Laboratory for their contributions and support. Also, I extend special thanks to the members of my committee, especially to my advisor Dr. Erin Strait for her mentorship and support. I am convinced that this experience has made me a wiser person and will continue to benefit me far into the future. I completely agree with the saying, “Tudo vem com um preço, mas é prazeroso no final.” This has been my story at Iowa State and I am very thankful.