Quantitative real-time polymerase chain reaction for detecting *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* in pen-based oral, tonsillar, and nasal fluids

João Carlos Gomes Neto¹², Leslie Bower¹, Barbara Z. Erickson¹, Chong Wang¹, Matthew Raymond¹³, Erin L. Strait¹⁴

¹Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 5011, USA
²352 Food Science Complex, University of Nebraska, Lincoln, NE 68583, USA
³Wisconsin National Primate Research Center, University of Wisconsin, Madison, WI 53715, USA
⁴Merck Animal Health, DeSoto, KS 66018, USA

*Mycoplasma (M.) hyorhinis* and *M. hyosynoviae* are pathogens known to cause disease in pigs post-weaning. Due to their fastidious nature, there is increased need for culture-independent diagnostic platforms to detect these microorganisms. Therefore, this study was performed to develop and optimize quantitative real-time PCR (qPCR) assays to rapidly detect *M. hyorhinis* and *M. hyosynoviae* in pen-based oral fluids as well as nasal and tonsillar fluids as proxies for samples used in swine herd surveillance. Two methods of genomic DNA extraction, automated versus manual, were used to compare diagnostic test performance. A wean-to-finish longitudinal study was also carried out to demonstrate the reproducibility of using pen-based oral fluids. Overall, pen-based oral and tonsillar fluids were more likely to be positive for both types of bacteria whereas only *M. hyorhinis* was detected in nasal fluids. DNA extraction protocols were shown to significantly influence test result. Although the initial detection time somewhat differed, both organisms were repeatedly detected in the longitudinal study. Overall, this study evaluated two qPCR methods for rapid and specific detection of either mycoplasma. Results from the present investigation can serve as a foundation for future studies to determine the prevalence of the two microorganisms, environmental load, and effectiveness of veterinary interventions for infection control.

**Keywords:** *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae*, real-time PCR, swine

**Introduction**

*Mycoplasma (M.) hyosynoviae* and *M. hyorhinis* are ubiquitous pathogens of swine known to cause arthritis and polyserositis, respectively, in pigs post-weaning [7,30,33]. Transmission of both pathogens presumably occurs either vertically or horizontally after initial exposure [14,19,35]. *M. hyorhinis* is more likely to be found in pigs immediately following weaning [21,35]. After initial colonization of the upper respiratory tract, bacteremia may occur leading to the development of polyarthritis, polyserositis, and potentially pneumonia [10,17,26]. *M. hyosynoviae* primarily colonizes the tonsils of weaned pigs but clinical arthritis only develops around 3 to 6 months of age [13,25,29].

Despite our understanding of the clinical disease, the true prevalence, incidence, and overall dynamics of infection by these mycoplasmas in swine populations is relatively unknown. Given the re-emergence of *Mycoplasma*-associated clinical arthritis that impacts both animal well-being and the profitability of clinically-affected populations [11], more epidemiological data will be needed to effectively implement disease control strategies. Due to the difficulty with culturing these species of mycoplasma, development of rapid, accurate, and culture-independent methods for quantifying these pathogens in multiple sample matrices is crucial. Therefore, the current study was conducted to establish quantitative real-time PCR (qPCR) assays and test the ability of these methods to detect both *M. hyosynoviae* and *M. hyorhinis* in pen-based oral fluid samples along with nasal and tonsillar fluids as proxies for diagnostic specimens commonly used in swine herd screening.
In addition, this study also verified the effects of an automated and a manual method of DNA extraction commonly performed in veterinary diagnostic laboratories on test result interpretation.

**Materials and Methods**

**Genomic DNA extraction protocols**

As a part of the diagnostic test validation, two distinct methods for total DNA extraction from pen-based oral, nasal, and tonsillar fluids were compared: magnetic beads (MB, automatic) and spin column (SC, manual). The MB (MagMAX Total Nucleic Acid Isolation Kit; Applied Biosystems, USA) and SC (High Pure PCR Template Preparation Kit; Roche, USA) DNA extraction protocols were performed according to the manufacturers’ instructions. For the MB procedure, DNA was extracted using a semi-automated nucleic acid purification system (KingFisher 96 magnetic particle processor; Thermo Fisher Scientific, USA). All DNA samples were frozen at $-20\,^\circ\mathrm{C}$ until qPCR analysis.

**Primer selection and design**

The *M. hyosynoviae*-specific primer pair, herein called “Lauerman qPCR,” was used for validation. This primer set was originally presented by Lauerman [18], but its validation and diagnostic test performance were never formally published. These primers were used in the current study for validation and verification of diagnostic performance without discarding the initial primer pair development. A new primer set was produced to detect *M. hyorhinis*. All primers used for this study targeted sequences within the hypervariable region of the 16S ribosomal DNA (rDNA) of the bacteria. For initial design and confirmation of specificity across all swine mycoplasmas, the 16S partial sequences for swine mycoplasmas were aligned. DNA sequence alignment was performed using a ClustalW algorithm available with commercially available software (DNASTAR Lasergene software ver. 8.0; DNASTAR, USA). The Lauerman qPCR primer sequences were 5’-CAGT TGAGGAAATGCAACTG-3’ (forward) and 5’-TAGCTGCG TCAGTGATTGG-3’ (reverse). The *M. hyorhinis* qPCR primer sequences were 5’-GCATGTGGAACGGATGTAAGCAAT-3’ (forward) and 5’-TGAAGCTGTGAAGCTCCTTATTA CTC-3’ (reverse). Specificity of the two primer pairs was subsequently re-confirmed in silico by checking them against a bacterial ribosomal database [4] and a probeCheck platform [23].

**qPCR**

qPCR specific for *M. hyosynoviae* and *M. hyorhinis* was performed using a fluorescence-based assay consisted of 2× commercial master mix (QuantiTect SYBR Green PCR Master Mix; Qiagen, USA) at a final concentration of 1×, each forward and reverse primer (0.4 μM final concentration), 2.5 μL template DNA, and nuclease-free water (up to 25 μL of the total reaction volume). All qPCR assays were performed with a commercial platform (ABI 7500 Fast Real-time PCR system; Life Technologies, USA). The assays were run under the following conditions: 15 min at 95°C followed by 45 cycles of 15 sec of denaturing at 94°C, 30 sec of annealing at 63°C (*M. hyosynoviae*) or 59°C (*M. hyorhinis*), and 30 sec of extension at 72°C; and a final melt curve from 95°C to 59°C. Assays for *M. hyosynoviae* and *M. hyorhinis* were performed in separate reactions given the difference in annealing temperature. All samples were tested in duplicate. In the case of discordant results (i.e., one positive and one negative), the sample was re-tested twice to achieve a consensus.

**qPCR data interpretation**

Analysis of the cycle threshold (Ct) value was performed using on-board qPCR software (ABI 7500 Fast software; Life Technologies) by setting the threshold manually at 0.04 with the baseline set from cycles 3 ~ 15. A sample was considered positive for *M. hyosynoviae* if the amplification curve did not exceed a Ct value of 35 and had a melting temperature ($T_m$) of 81.5 ± 0.5°C. Samples were positive for *M. hyorhinis* only if the limit of detection did not exceed a Ct value of 38 and had a $T_m$ of 75.9°C ± 0.5°C. Samples collected after an experimental challenge with all four major swine mycoplasmas, including the two tested in the present study, were used as in-house positive and negative controls for pen-based oral, nasal, and tonsillar fluids [12].

**Assay specificity**

To measure the specificity of each primer pair, a collection of bacterial isolates commonly found in swine that had been previously identified was utilized [34]. The collection included both mycoplasmas and non-mycoplasma species present in the swine respiratory tract, joints, or peritoneal cavities. This panel was specifically chosen due to its representativeness of known swine pathogens that could possibly be a confounding factor for development of the assays. More importantly, the panel had also been previously used to develop *M. hyopneumoniae*-specific PCR primers, therefore indicating its validity.

**Analytical sensitivity**

Two plasmids were synthesized (Integrated DNA Technologies, USA) as quantitative standards. One contained the 160-base pair target region of *M. hyorhinis* 16S rDNA and the other contained the 392-base pair target region of *M. hyosynoviae* 16S rDNA. Insert sequences were synthesized into pIDTSMART plasmids containing restriction sites flanking each end of the insert sequences. Both plasmids were linearized and prepared in ten 10-fold serial dilutions ranging from $1 \times 10^0$ to 1 copy for sensitivity testing and standard curve analysis. Standard curves were generated in triplicate to determine the limit of detection.
and efficiency of each reaction. In order to estimate the total number of bacterial genome copies (genome equivalents), one copy of the 16S rDNA gene was used for either mycoplasma by taking into consideration data available from the ribosomal RNA database [20]. DNA isolated from serially diluted cultures of either mycoplasma was used to confirm results predicted by the plasmid standard curves. Mycoplasma culture procedures were performed as previously described [31]. Ultimately, the plasmid standard curves were used throughout the study for consistent quantification of bacterial load in the diagnostic specimens.

**Diagnostic specimens for qPCR performance testing**

For diagnostic performance verification using field samples that can serve as proxies for swine herd screening, five commercial finishing sites that received pigs from a common sow herd were selected for sampling. Each site had 2,000 to 4,000 18- to 24-week-old pigs housed in one or two barns. Sites were selected based on the clinical history reported by the consultant veterinarian of Mycoplasma-associated arthritis in previous groups of pigs produced from the same groups of sows in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterinary diagnostic laboratory and used as a defining criterion for the presence of both pathogens in piglets produced in the herd. The clinical history was confirmed by the Iowa State Veterans
Overall, the spin column method of DNA extraction increased test sensitivity for both \textit{M. hyosynoviae} and \textit{M. hyorhinis} (Table 1, Figs. 1 and 2).

**Longitudinal study**

Results of the longitudinal study revealed distinct patterns of detection for \textit{M. hyosynoviae} and \textit{M. hyorhinis} in serially collected pen-based oral fluids. \textit{M. hyosynoviae} was not found until 88 DPP when only 40\% of the pens were positive (2 of 5) with all samples testing positive thereafter. \textit{M. hyorhinis} was initially detected in 60\% of the samples on day 28 DPP (3 of 5) and subsequently in all five pens. Quantification of mycoplasma load in oral fluids showed that the overall bacterial load was low over time (Fig. 3).

**Discussion**

\textit{M. hyosynoviae} and \textit{M. hyorhinis} can be isolated from the tonsils and nasal cavity of experimentally inoculated and naturally exposed animals using standard culture procedures [9,15,16,24]. However, standard culture techniques are often complicated by overgrowth of other fast-growing bacteria [8,22,35]. Therefore, qPCR assays were developed and validated in the current study to be used as high-throughput and rapid diagnostic alternatives for detecting and quantifying \textit{M. hyosynoviae} and \textit{M. hyorhinis} in specimens used as proxies for field samples routinely used in swine population screening.

Since \textit{M. hyosynoviae} and \textit{M. hyorhinis} are presumably long-term colonizers of the upper respiratory tract of pigs, it was hypothesized that pen-based oral, tonsillar, and nasal fluids would yield positive test results provided that these organisms were known to be circulating in the population [3,13,17,32]. This criterion was established while taking into consideration

\begin{table}
\centering
\begin{tabular}{llllll}
\hline
\textbf{Diagnostic specimen} & \textbf{Number of positive results for the relevant extraction protocol}\textsuperscript{1} & & & & \\
  & \textbf{MB Pos (%)} & \textbf{SC Pos (%)} & \textbf{Kappa coefficient}\textsuperscript{2} & \textbf{p value McNemar’s test}\textsuperscript{3} & \\
\hline
\textit{M. hyosynoviae} & & & & & \\
TS (n = 49) & 5 (10\%) & 18 (37\%) & 0.327 & 0.0009 & \\
OF (n = 60) & 0 (0\%) & 17 (28\%) & 0 & 0.0001 & \\
NS (n = 81) & 0 (0\%) & 0 (0\%) & 1 & 1 & \\
\textit{M. hyorhinis} & & & & & \\
TS (n = 49) & 45 (92\%) & 47 (96\%) & 0.296 & 0.6171 & \\
OF (n = 60) & 22 (37\%) & 45 (75\%) & 0.324 & 0.0001 & \\
NS (n = 81) & 60 (74\%) & 77 (95\%) & -0.047 & 0.0005 & \\
\hline
\end{tabular}
\caption{Summary of \textit{Mycoplasma (M.) hyosynoviae} and \textit{M. hyorhinis} real-time polymerase chain reaction (qPCR) positive results (\%) according to diagnostic specimen and extraction protocol\textsuperscript{*}}
\end{table}

\textsuperscript{*The total number of tested samples per diagnostic specimen is indicated in parentheses. \textsuperscript{1}Pos (\%) represents the number and percentage of positive samples. The genomic DNA extraction methods used in this study were magnetic beads (MB) and a spin column (SC). \textsuperscript{2}Kappa Cohen’s coefficient was used to compare the degree of agreement between the two genomic DNA extraction methods based on qPCR results. \textsuperscript{3}McNemar’s chi-square test was used to compare the percentage of agreement between the two genomic DNA extraction methods based on qPCR results (p < 0.05). TS: tonsil scraping fluid, NS: nasal swab fluid, OF: pen-based oral fluid.
Rapid detection of *Mycoplasma hyosynoviae* and *Mycoplasma hyorhinis* by qPCR

Fig. 2. *M. hyorhinis* load in tonsillar, pen-based oral, and nasal fluids according to two distinct methods of genomic DNA extraction (magnetic beads versus spin column). The box-and-whisker plot only includes samples that were positive in order to demonstrate the range of bacterial load among diagnostic specimens and DNA extraction protocols. Asterisks indicate significant differences for the log10 number of bacteria when comparing the two methods of DNA extraction ($p < 0.05$). The limit of detection for *M. hyorhinis*-specific qPCR is indicated in the figure.

Fig. 3. *M. hyosynoviae* and *M. hyorhinis* load in pen-based oral fluids serially sampled from wean-to-finish pigs in a longitudinal study. Sampling was performed for the same five pens initially selected. The box-and-whisker plot includes only samples that were positive in order to demonstrate the range of bacterial load in the pen-based oral fluids over time. The limit of qPCR detection for both types of mycoplasma is indicated in the figure.

that a herd with a clinical history of *Mycoplasma*-associated arthritis was selected for sampling. As shown in the present investigation, both *M. hyosynoviae* and *M. hyorhinis* could be detected in tonsillar and pen-based oral fluids whereas only *M. hyorhinis* was found in nasal fluids. To date, it is still unclear whether or not *M. hyosynoviae* has a differential tropism for tonsils compared to the nasal cavity, or if an ecological barrier exists that determines the inhabitance and ecology of these structures, or niches. It appears that *M. hyorhinis* is less likely to be influenced by anatomical location. While these results are intriguing, careful interpretation is warranted since confounding factors such as sampling, number of animals, true prevalence, dynamics of infection, and host immunity may alter data interpretation.

Comparison between two different genomic DNA extraction protocols performed in the current investigation was made based on a previous report in which significant differences were observed in the rate of porcine reproductive and respiratory syndrome virus detection in oral fluids using different methods [2]. Additionally, many veterinary diagnostic laboratories use automatic genomic DNA extraction procedures to expedite test results, thus increasing the relevance of the comparison here. In this study, the manual procedure using a spin column was shown to increase test sensitivity for both *M. hyosynoviae* and *M. hyorhinis*. As recently suggested [3], this step can affect detection of these mycoplasmas in pen-based oral fluids. As demonstrated here, this may be true for other specimens as well. Results of the present investigation suggest that the number of false negatives can be affected by the genomic DNA extraction protocol used depending on the study design and nature of sampling. Importantly, these findings should increase the awareness of veterinarians and diagnosticians to not only interpret the qPCR test results as is (e.g., cycle threshold values as positive or negative), but to incorporate the clinical history of the farm, specifically the expected prevalence, to determine the positive and negative predictive values of the test.

While previous research has demonstrated that a variety of other swine pathogens may be found in porcine oral fluids [1,3,5,6,28], the current study is the first to show that *M. hyosynoviae* can also be detected by qPCR in pen-based oral fluid samples. In our longitudinal study, delayed detection of either mycoplasma may have been caused by multiple factors such as protective circulating maternal antibodies, low rate of transmission, few infected animals at the time of weaning, or unknown host factors that confer temporary immunity. This is assuming that group-based sampling reflects the individual status of pen mates, which is yet to be proven. In summary, this study provided a new molecular approach to be used in veterinary diagnostics for specific quantification of two re-emerging swine pathogens. Our findings can serve as a foundation for future studies to understand the true prevalence, incidence, and impact of production interventions for controlling *M. hyosynoviae* and *M. hyorhinis* infection in pig populations.
Acknowledgments

The authors would like to thank Mr. Brent Pepin (College of Veterinary Medicine, Iowa State University, Ames, USA), Dr. Todd Wolf, and Mr. Kent Naughton for their help with the field studies. The authors also thank Dr. Jeffrey Zimmerman (College of Veterinary Medicine, Iowa State University, Ames, USA) for his help with the manuscript. This work was supported by the Veterinary Diagnostic Laboratory at the Iowa State University College of Veterinary Medicine, USA.

Conflict of Interest

There is no conflict of interest.

References

1. Bender JS, Shen HG, Irwin CK, Schwartz KJ, Opiressnig T. Characterization of *Erysipelothrix* species isolates from clinically affected pigs, environmental samples, and vaccine strains from six recent swine erysipelas outbreaks in the United States. Clin Vaccine Immunol 2010, 17, 1605-1611.
2. Chittick WA, Stensland WR, Prickett JR, Strait EL, Harmon K, Yoon KJ, Wang C, Zimmerman JJ. Comparison of RNA extraction and real-time reverse transcription polymerase chain reaction methods for the detection of *Porcine reproductive and respiratory syndrome virus* in porcine oral fluid specimens. J Vet Diagn Invest 2011, 23, 248-253.
3. Clavijo MJ, Oliveira S, Zimmerman J, Rendahl A, Rovira A. Field evaluation of a quantitative polymerase chain reaction assay for *Mycoplasma hyorhinis*. J Vet Diagn Invest 2014, 26, 755-760.
4. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM. Ribosomal database project: data and tools for high throughput rRNA analysis. Nucl Acids Res 2014, 42, D633-642.
5. Costa G, Oliveira S, Torrison J. Detection of *Actinobacillus pleuropneumoniae* in oral-fluid samples obtained from experimentally infected pigs. J Swine Health Prod 2012, 20, 78-81.
6. Detmer SE, Patnayak DP, Jiang Y, Gramer MR, Goyal SM. Detection of *Influenza A virus* in porcine oral fluid samples. J Vet Diagn Invest 2011, 23, 241-247.
7. Friis NF. Mycoplasmas cultivated from the respiratory tract of Danish pigs. Acta Vet Scand 1971, 12, 69-79.
8. Friis NF, Ahrens P, Larsen H. *Mycoplasma hyosynoviae* isolation from the upper respiratory tract and tonsils of pigs. Acta Vet Scand 1991, 32, 425-429.
9. Gois M, Cerny M, Rozkosny V, Sovadina M. Studies on the epizootiological significance of some species of mycoplasma isolated from nasal swabs and lungs of pigs. Zentralbl Veterinarmed B 1969, 16, 253-265.
10. Gois M, Pospisil Z, Cerny M, Mrva V. Production of pneumonia after intranasal inoculation of gnotobiotic piglets with three strains of *Mycoplasma hyorhinis*. J Comp Pathol 1971, 81, 401-410.
11. Gomes Neto JC, Gauger PC, Strait EL, Boyes N, Madson DM, Schwartz KJ. Mycoplasma-associated arthritis: critical points for diagnosis. J Swine Health Prod 2012, 20, 82-86.
12. Gomes Neto JC, Strait EL, Raymond M, Ramirez A, Minion FC. Antibody responses of swine following infection with *Mycoplasma hypopneumoniae*, *M. hyorhinis*, *M. hyosynoviae* and *M. flocculare*. Vet Microbiol 2014, 174, 163-171.
13. Hagedorn-Olsen T, Nielsen NC, Friis NE. Induction of arthritis with *Mycoplasma hyosynoviae* in pigs: clinical response and re-isolation of the organism from body fluids and organs. Zentralbl Veterinarmed A 1999, 46, 317-325.
14. Hagedorn-Olsen T, Nielsen NC, Friis NE, Nielsen J. Progression of *Mycoplasma hyosynoviae* infection in three pig herds. Development of tonsillar carrier state, arthritis, and antibiotics in serum and synovial fluid in pigs from birth to slaughter. Zentralbl Veterinarmed A 1999, 46, 555-564.
15. Harris DL, Ross RF, Switzer WP. Incidence of certain microorganisms in nasal cavities of swine in Iowa. Am J Vet Res 1969, 30, 1621-1624.
16. Kawamura S, Yamamoto K, Ogata M. Isolation of *Mycoplasma hyosynoviae* and other mycoplasmas from the respiratory tracts of pigs by aerobic and anaerobic cultivation. Nihon Juigaku Zasshi 1982, 44, 811-814.
17. Kobisch M, Friis NF. Swine mycoplasmoses. Rev Sci Tech 1996, 15, 1569-1605.
18. Lauerman LH. Mycoplasma PCR assays. In: Lauerman LH (ed.). Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases. pp. 41-42, American Association of Veterinary Laboratory Diagnostics, Auburn, 1998.
19. Lauritsen KT, Hagedorn-Olsen T, Friis NE, Lind P, Jungersen G. Absence of strictly age-related resistance to *Mycoplasma hyosynoviae* infection in 6-week-old pigs. Vet Microbiol 2008, 130, 385-390.
20. Lee ZM, Bussema C 3rd, Schmidt TM. rrdDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res 2009, 37, D489-493.
21. Lin JH, Chen SP, Yeh KS, Weng CN. *Mycoplasma hyorhinis* in Taiwan: diagnosis and isolation of swine pneumonia pathogen. Vet Microbiol 2006, 115, 111-116.
22. Lowe BA, Marsh TL, Isaacs-Cosgrove N, Kirkwood RN, Kiupel M, Mulks MH. Microbial communities in the tonsils of healthy pigs. Vet Microbiol 2011, 147, 346-357.
23. Loy A, Arnold R, Tischler P, Rattei T, Wagner M, Horn M. probeCheck – a central resource for evaluating oligonucleotide probe coverage and specificity. Environ Microbiol 2008, 10, 2894-2898.
24. Makhonon M, Tummaruk P, Thongkamkoon P, Thawawongnuwech R, Prapasarakul N. Comparison of detection procedures of *Mycoplasma hypopneumoniae*, *Mycoplasma hyosynoviae*, and *Mycoplasma hyorhinis* in lungs, tonsils, and synovial fluid of slaughtered pigs and their distributions on Thailand. Trop Anim Health Prod 2012, 44, 313-318.
25. Nielsen EO, Nielsen NC, Friis NE. *Mycoplasma hyosynoviae* arthritis in grower-finisher pigs. J Vet Med A Physiol Pathol Clin Med 2001, 48, 475-486.
26. Palzer A, Ritzmann M, Wolf G, Heinritz K. Associations between pathogens in healthy pigs and pigs with pneumonia. Vet Rec 2008, 162, 267-271.
27. Prickett J, Simer R, Christopher-Hennings J, Yoon KJ, Evans RB, Zimmerman JJ. Detection of porcine reproductive and respiratory syndrome virus infection in porcine oral fluids samples: a longitudinal study under experimental conditions. J Vet Diagn Invest 2008, 20, 156-163.
28. Prickett JR, Johnson J, Murtaugh MP, Puvanendiran S, Wang C, Zimmerman JJ, Opriessnig T. Prolonged detection of PCV2 and anti-PCV2 antibody in oral fluids following experimental inoculation. Transbound Emerg Dis 2011, 58, 121-127.
29. Ross RF. Predisposing factors in Mycoplasma hyosynoviae arthritis in swine. J Infect Dis 1973, 127 (Suppl), S84-86.
30. Ross RF, Duncan JR. Mycoplasma hyosynoviae arthritis of swine. J Am Vet Med Assoc 1970, 157, 1515-1518.
31. Ross RF, Karmon JA. Heterogeneity among strains of Mycoplasma granularum and identification of Mycoplasma hyosynoviae, sp. n. J Bacteriol 1970, 103, 707-713.
32. Ross RF, Spear ML. Role of the sow as a reservoir of infection for Mycoplasma hyosynoviae. Am J Vet Res 1973, 34, 373-378.
33. Ross RF, Switzer WP, Duncan JR. Experimental production of Mycoplasma hyosynoviae arthritis in swine. Am J Vet Res 1971, 32, 1743-1749.
34. Strait EL, Madsen ML, Minion FC, Christopher-Hennings J, Dammen M, Jones KR, Thacker EL. Real-time PCR assays to address genetic diversity among strains of Mycoplasma hyopneumoniae. J Clin Microbiol 2008, 46, 2491-2498.
35. Thacker EL, Minion FC. Mycoplasmosis. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW (eds). Diseases of Swine. 10th ed. pp. 779-797. Wiley-Blackwell, Ames, 2012.