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Temporal patterns of colonization and infection with *Mycoplasma hyorhinis* in two swine production systems in the USA

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ABSTRACT

Control of *Mycoplasma hyorhinis* (*M. hyorhinis*) associated disease is currently hindered by limited knowledge of the epidemiology and ecology of this organism. A prospective longitudinal investigation was conducted to determine the dynamics of *M. hyorhinis* colonization in two swine production systems. In each system (A, B), 51 young sows (parities 1, 2) and 56 older sows (> parity 2) were selected at farrowing and tested by qPCR of nasal swabs and for antibodies by serum ELISA. From each sow, a piglet was randomly selected, and nasal and serum samples were collected at birth, weaning, and 10 days post-weaning. Two further samplings were performed in the nursery and finishing stages during the high-risk periods for *M. hyorhinis*-associated disease, and 12 pigs were euthanized and necropsied at these later sampling events. The prevalence of *M. hyorhinis* colonization in sows was low (< 5%). No associations were found between sow parity or sow serum titer and piglet nasal colonization at either birth or weaning. In contrast to the low prevalence (0.95–2.70%) observed in piglets pre-weaning, most pigs became colonized during the first four weeks after weaning and remained positive throughout the nursery and finishing stages. The detection of *M. hyorhinis* in oral fluids followed similar patterns as those observed using nasal swabs. ELISA results showed decreased detection of maternal antibodies at around 3 weeks of age and a subsequent increase after natural exposure. The role of *M. hyorhinis* in polyserositis and arthritis was demonstrated in these two herds. Establishing the temporal dynamics of exposure and infection with *M. hyorhinis* in pigs will enable more strategic implementation of intervention strategies in affected herds.

1. Introduction

Mycoplasma hyorhinis (*M. hyorhinis*) belongs to the mollicutes class, which comprises more than 100 mycoplasma species that infect humans and animals (Razin et al., 1998). Mollicutes are the simplest forms of life that can replicate in cell-free media and are typically closely adapted to a particular host species (Citti and Blanchard, 2013). The natural habitat for *M. hyorhinis* are the mucous membranes of the upper respiratory tract and tonsils of pigs, and it can frequently be isolated from lungs of healthy pigs (Friis and Feenstra, 1994). This wall-less bacterium is one of the causative agents of polyserositis and arthritis in post-weaning pigs, and it has also been linked to eustachitis (Morita et al., 1999) and pneumonia (Gois et al., 1971; Poland et al., 1971). The detection of *M. hyorhinis* is mainly achieved through PCR or bacterial isolation. Although several serological assays have been developed to detect antibodies against this pathogen, these are not commercially available (Poland et al., 1971; Gois et al., 1972; Neto et al., 2014).

Knowledge of the epidemiology of *M. hyorhinis* in commercial swine herds is minimal. *M. hyorhinis* can be transmitted through direct contact between infected and naïve pigs. Recently, the prevalence of colonization in pigs of different ages from three commercial swine herds was estimated (Clavijo et al., 2017). In that cross-sectional study, the prevalence of colonization in sows and pre-weaning piglets was relatively low (< 10%) compared with prevalence in pigs after weaning, which

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Table 1
Description of swine production systems.

	System A	System B
Breeding herds		
Herd size-	4700	6000
Herd Location (state)	MN	MN
Production type	Farrow-to-wean	Farrow-to-wean
Gilt source/management	Internal multiplication	Internal multiplication
PRRS status	Negative	Positive unstable
M. hyopneumoniae status	Positive	Positive
Antibiotic treatment:		
Piglets	Birth: long acting penicillin, spot treatment penicillin and gentamicin	Draxxin at weaning
Sows	Short acting Penicillin	Spot treatment penicillin
Vaccine protocol:		
Piglets	Day 4- autogenous bacterial: *S. suis, H. parasuis, E. rhusiopathiae*, *A. suis* and PCV2 Weaning: autogenous bacterial + *M. hyopneumoniae*,	Weaning: *M. hyopneumoniae*, *E. rhusiopathiae, Salmonella sp. and L. intracellularis,*
Sows	Influenza, autogenous bacterial, *Leptospira sp.*, Parvovirus, *E. Rhusiopathiae*, *C. perfringens, E. coli,* PCV2 and *M. hyopneumoniae*	Influenza, *Leptospira sp.*, Parvovirus, *E. Rhusiopathiae*
Weaning age	18-21 days	17-22 days
Pre-weaning mortality rate (%)	10-12	10-12
Nurseries		
Location	MN	MN
Weaned pig facility	Wean-to-finish	Traditional nursery
Pig Flow	Multisource all-in all-out (AIAO)	Single source flow all-in all-out (AIAO)
Capacity/per site	4000	6000
Stocking density (m^2/animal)	0.3	0.3
Number of pigs per pen	50	25
Antibiotic treatments	Tiamulin/Chlortetracycline (feed), amoxicillin and neomycin (water). Aspirin in water	Spot treatment penicillin, amoxicillin aspirin (water). Pulses of Chlortetracycline (feed) and tetracycline (water)
Vaccination protocol	*M. hyopneumoniae*, PCV2, *E. rhusiopathiae, Salmonella spp. and L. intracellularis,*	PCV2, *M. hyopneumoniae*, *E. rRhusiopathiae*, *Salmonella spp. and L. intracellularis,*
Mortality rate (%)	3	2-4
Finishers:		
Location	MN	MN
Finishing facility	Multisource Grow-finish	Single source grow-finish
Pig Flow	all-in all-out	all-in all-out
Capacity/per site	2000	2300
Stocking density (m^2/animal)	0.7	0.7
Number of pigs per pen	25	25
Antibiotic treatments	Chlortetracycline (feed), citric acid and aspirin (water)	First two weeks: Tiamulin/ Chlortetracycline (feed)

reached 100% by the end of the nursery phase. The data indicated that a low proportion of young positive sows yielded colonized piglets which likely become the source of infection for other pigs after weaning. A longitudinal study involving repeated sampling of individual pigs was conducted to describe the dynamics of *M. hyorhinis* exposure and disease in cohorts of growing pigs. The specific objectives of this study were (1) to determine the prevalence and incidence of *M. hyorhinis* in naturally colonized pigs over time and (2) to evaluate the relationships between sow colonization and serological status with piglet colonization.

2. Materials and methods

2.1. Study design

All protocols of pig care and management were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC protocol number 1006A84138). The study was a prospective cohort study in which a nasal swab and a blood sample were collected from sows shortly after farrowing. Sows were stratified by parity into 2 groups of younger [parity 1 (p1) and parity 2 (p2)] and older [parity 3 (p3) and above] sows. From each litter born to selected sows, one piglet was selected and tagged shortly after birth. Nasal swabs and blood samples were collected from the selected piglets multiple times during their life. In each system, *M. hyorhinis*-associated disease was investigated by means of necropsy of pigs during periods of elevated mortality in the nursery and finishing stages (Supplemental Fig. 1).

2.2. Selection of herds

Herd inclusion criteria were: (1) three-site production systems, (2) breeding herd of at least 1500 sows, (3) recent history cases of *M. hyorhinis*-associated disease diagnosed by PCR or culture, (4) no current vaccination against *M. hyorhinis,* (5) location within 200 miles of the University of Minnesota Saint Paul Campus, and (6) willingness to collaborate in a long-term project. Seven herds that fulfilled conditions 1, 2 and 3 were evaluated as candidates for the study. One system was excluded due to the use of an *M. hyorhinis* autogenous vaccine, and two herds were eliminated due to distance from the University of Minnesota. Two other eligible herds could not be included as either the veterinarian or the owner decided not to participate. The two remaining herds fulfilled all the inclusion criteria and were enrolled in the study. These herds (A and B) belonged to two different commercial pork production companies located in swine dense regions in southern Minnesota and were representative of other commercial herds in the USA, in terms of housing, management, female replacement rate, mortality rates, weaning age and antimicrobial and vaccination programs. Prior to initiating the longitudinal sampling protocol, the presence of *M. hyorhinis* disease was confirmed in both systems by necropsies of clinically affected pigs in the respective nurseries.

2.3. Herd descriptions

2.3.1. Herd A

The farrow-to-wean breeding farm housed 4700 sows in buildings connected through hallways, including16 farrowing rooms managed

all-in-all-out (AIAO) by room. Gestating sows were housed in individual crates and gilts were housed in pens. The replacement gilts were obtained using internal multiplication, with replacements introduced in groups every month into gilt development units (GDUs), then from the GDUs into the breeding barn every week. Piglets were weaned at 18–21 days and two groups of approximately 1300 pigs were transported to off-site growing farms each week. At the time of the study, the breeding farm was weaning pigs negative for porcine reproductive and respiratory syndrome virus (PRRSV). Pigs were transported to off-site, double-stocked, wean-to-finish facilities comprising one building and two rooms with 40 pens each. These sites commingled pigs originating from multiple breeding herds within the same company. All pigs in the study cohort, however, were housed in the same room and were separated from pigs from other sources by a solid wall and a hallway. After seven weeks post-placement in the nursery, the study pigs were transported to an AIAO multisource finishing farm. All pigs were housed in two buildings with two rooms per building separated by a solid wall. Post-weaning facilities commingled pigs from five different sow herds (Table 1). Study pigs were housed together with their sow farm cohort and were not commingled with pigs originating from other sow farms.

2.3.2. Herd B

The breeding farm housed 6000 sows in two large interconnected buildings with 21 farrowing rooms managed AIAO by room. All sows and gilts were housed in crates during all production stages. Piglets were weaned at 17–21 days of age two to three times per week and transported to off-site nursery farms. The breeding farm was weaning PRRSV-positive pigs at the time of the study. The single-source nursery facilities (meaning all pigs originated from the same breeding farm) were comprised of two barns housing 6000 pigs in six or 12 rooms with a capacity of 1000 or 500 pigs, respectively. There were between 20 and 40 pens in each nursery room. The pigs were managed AIAO by barn and remained at these facilities for seven weeks, after which they were transported to single-source AIAO finishing sites comprised of two 1,000-head barns or one 300-head barn (Table 1).

2.4. Sampling protocol

2.4.1. M. hyorhinis nasal colonization

2.4.1.1. Breeding farms. To identify possible differences of *M. hyorhinis* colonization patterns in pigs born from sows of different parity, the sows were stratified into young (p1 and p2) and old (p3 and above). A total of 51 young sows and 56 old sows were selected shortly after farrowing (Supplemental Fig. 1). For both breeding farms, parturition was induced one day early (115 days) to guarantee the sample size needed on the first sampling day. Sample size was estimated based on previous data indicating a higher prevalence of *M. hyorhinis* qPCR-positive younger sows (20%) compared to older sows (2.5%) (Clavijo et al., 2017). Sample size estimation to compare proportions assumed a relative risk of 8, a confidence level of 95%, and a power of 80% (Sergeant ESG., 2019). A bilateral nasal swab and a blood sample were collected from each sow selected. One piglet per litter was selected by simple randomization from each previously selected sow for a total of 107 piglets. Briefly, for each litter, a random number was generated using an online random number generator from 1 to 15 (www.random.org)). At the time of the sampling, one person blinded to the list enumerated piglets, and when the random number was called out, the piglet was selected. Piglets were ear tagged, and a bilateral nasal swab and a blood sample were obtained within 24 h after birth. Nasal swabs were collected again from each pig at weaning. For Breeding farm B, a blood sample was also obtained at the same time (Supplemental Fig. 1). Piglets selected for this study were not cross-fostered and no piglets were added to the litters of study sows. Therapeutic antibiotic treatments, parity of sows, and gender of piglets were recorded. None of the monitored piglets or sows received preventive antibiotic treatments in the farrowing room.

In addition, the presence of *M. hyorhinis* in the environment of suckling piglets was investigated as a potential source of indirect transmission of *M. hyorhinis*. Cloth pads (Swiffer® cloths) have been used to detect PRRSV in the environment (Pitkin et al., 2009). A total of twelve cloth pads (Swiffer® cloths), previously soaked in PBS, were used to sample the floors, walls, bars, and feeders of farrowing crates with pigs (n = 10) and without pigs (n = 2) in each herd at weaning. Sampled crates housing study pigs were chosen by simple randomization.

2.4.1.2. Growing pig sites. A nasal swab and blood sample were collected from each tagged pig 10 days after placement into the nursery. Each pig was sampled again on two additional occasions, once later in the nursery stage and once during the finishing stage. These latter sampling events were scheduled to coincide with the peak of polyserositis/arthritis/pneumonia syndromes in the nursery and finishing stages based on clinical signs reported by the herd veterinarians and historic mortality records. Twelve pigs from each herd were euthanized and necropsied for post-mortem investigation of the causes of disease (Supplemental Fig. 1).

2.4.2. Oral fluids

The utility of oral fluids for *M. hyorhinis* detection was evaluated by collecting a set of oral fluid samples at each post-weaning sampling event (described in previous section). In each herd, between seven and 12 oral fluid samples were collected from pens containing the pigs in the study cohort. Non-bleached cotton ropes were suspended at the pigs' shoulder level in each pen. All tagged pigs and their pen mates had access to the rope for approximately 45 min, after which the rope was transferred into a plastic bag, and the oral fluid was removed from the rope by compressing the wet portion. The expressed liquid was then placed into a plastic sterile tube and stored at −80 °C until testing for *M. hyorhinis* by PCR (Prickett et al., 2008).

2.4.3. Clinical signs and postmortem observations

The attending veterinarians considered the current clinical picture, as well as previous mortality rates, to estimate the ideal time for the necropsy investigations. A total of 10 tagged pigs with clinical signs indicative of polyserositis (reluctance to move, cough, lameness, dyspnea, and fever) and two clinically healthy tagged pigs were humanely euthanized, and necropsies were performed. Pleural, pericardial, and synovial surfaces were swabbed and tested for *M. hyorhinis* by qPCR, for *Haemophilus parasuis* by PCR, and for aerobic bacteria by bacterial culture. In addition, liver and lung tissue samples were used for aerobic bacterial culture. A set of aseptically collected samples from lung, heart, spleen, kidney, liver, and inguinal lymph node were obtained from each pig and fixed in 10% formalin for histological assessment. PRRSV and porcine circovirus type 2 (PCV2) PCRs were performed on a tissue pool containing lung, spleen, and inguinal lymph node from each individual pig. Bronchial swabs were tested for *Mycoplasma hyopneumoniae* and *M. hyorhinis* by PCR, and a lung sample was utilized for detection of *Influenza A* virus (IAV) by PCR. In addition, a subset of the monitored pigs (9) from Herd A was sampled at slaughter. From each pig, a tonsil and a joint swab were collected. Due to the logistics at the slaughter plant, nasal swabs could not be collected.

2.5. Diagnostic testing

2.5.1. Bacteriological culture

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

Table 2
Prevalence of *M. hyorhinis* infection, parity, bacterial load and serum titer in sows.

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

After 2–14 days incubation at 37 °C, isolates were stored at −80 °C following confirmation of growth in agar media.

2.5.2. Sample processing and quantitative PCR

Oral fluids and nasal, serosal, and bronchial swabs collected from sows and pigs were stored at −20 °C until testing was performed. Swabs were immersed in 0.5 ml of Dulbecco's phosphate buffered saline (DPBS) (Sigma-Aldrich, St Louis, MO). The suspension was vortexed and centrifuged, and DNA was extracted from the pellet using the DNeasy Blood & Tissue kit (Qiagen, Germantown, MD). All samples were tested by a TaqMan® qPCR targeting the 16S rRNA gene of *M. hyorhinis* (Clavijo et al., 2014). The qPCR reaction was prepared in a volume of 25 µl consisting of 5 µl of the template DNA, 12.5 µl of Master Mix solution, 1 µl of ROX dye, 0.125 µl of each 40 µM reverse and forward primers, 1.25 µl of 5 µM TaqMan probe® and 5.0 µl of DNAse free H_20. Reactions were carried out in the ABI 7500 fast real-time PCR system (Life Technologies, Grand Island, NY) at 95 °C for 3 min, 35 cycles of 95 °C for 15 s and 54 °C for 50 s. Individual samples were run in duplicate, and each run contained a negative and positive extraction control as well as a PCR negative control (H_2O).

2.5.3. Serology

Blood samples were collected by jugular venipuncture, and the serum was separated by centrifugation and stored at -20 °C until testing. An ELISA protocol to detect *M. hyorhinis* was developed and optimized as follows: High protein-binding capacity polystyrene 96-well ELISA plates (MaxiSorp™, NUNC, Roskilde, Denmark) were coated overnight with whole-cell antigen from a US clinical isolate of *M. hyorhinis* diluted 1:400 in carbonate/bicarbonate buffer (Sigma Aldrich, St. Louis, MO, USA) at 4 °C and blocked with 5% fish gelatin (Sigma Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) (Gibco, Carlsbad, CA, USA) containing 0.05% Tween 20 (Sigma Aldrich, St. Louis, MO) at 37 °C for 1 h. Serum samples were prepared as two-fold serial dilutions from 1:100 to 1:3200 in blocking buffer using a robotic liquid handler (Biomek 3000, Beckman Coulter, Brea, CA, USA) and added to the ELISA plates along with positive and negative control sera diluted 1:100 in blocking buffer. Sealed plates were incubated at 37 °C for 1 h followed by three washes with PBS containing 0.05% Tween 20. A horseradish peroxidase-conjugated goat anti-swine IgG(H + L) antibody (Kierkegaard and Perry Laboratories, Gaithersburg, MA, USA) was diluted 1:1000 in blocking buffer, added to each well, and incubated for 1 h at 37 °C. Plates were then washed as described and developed with TMB peroxidase solution (Kierkegaard and Perry Laboratories). Optical density (OD) was measured at 405 and 490 nm (Spectramax Plus, Molecular Devices, Sunnyvale, CA, USA). For each sample and each dilution, a corrected OD value was calculated by subtracting the OD obtained at 490 nm from the OD obtained at 405 nm. Samples were considered positive when higher than the corrected OD for the negative control plus 2 standard deviations. The titer of each sample was calculated as the maximum dilution yielding a positive result.

2.6. Statistical analysis

Incidence rate (new cases per pig days at risk) is defined as the number of new cases in a population per unit of animal-time during a given time period (Dohoo et al., 2003). Since the actual mortality days were unknown, it was assumed that mortalities occurred at the mid-point time. Incidence rates and confidence intervals were calculated for each time interval between successive samplings (4-time intervals per herd). The equation for incidence rate was:

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

Apparent prevalence at each sampling event was estimated as the number of positive pigs divided by the total number of pigs sampled. The 95% confidence intervals for each prevalence estimate and for the proportions of positive oral fluid samples were calculated using the Wilson's score method (Wilson, 1927). Bacterial load (expressed as geq/swab or geq/mL of oral fluid), and serology data (expressed as serum titer) were entered into Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) and exported to R for statistical analysis on $\log_{10}$ transformed data (R Core Team, 2013). Graphical descriptive analysis was carried out using dot plots and box plots. The proportions of positive sows between groups (young and old) and the proportions of positive piglets from young and old sows were compared using a Fisher's exact test.

3. Results

3.1. M. Hyorhinis nasal colonization

3.1.1. Breeding herd

M. hyorhinis was detected in 5/107 sows (4. 7%, 95% CI: 2–10.5%) in Herd A and 2/110 sows (1.8%, 95% CI: 0.5–6.4%) in Herd B (Table 2). The median bacterial load for sows in Herd A was 3.8 $\log_{10}$ geq/swab (range 3.6–4.2 $\log_{10}$ geq/swab), while in Herd B, the bacterial load for the two positive sows was 4.6 and 4.7 $\log_{10}$ geq/swab. There was no statistical difference between the proportion of positive young sows and the proportion of positive old sows. In Herd B, however, positive results were only found in parity 1 sows. In Herd A, the parities of the positive sows were 2 (n = 2), 3 (n = 1), and 6 (n = 2). All positive piglets at birth originated from negative sows while one of three positive piglets at weaning originated from positive sows at farrowing in Herd B. There was no significant difference between the proportions of positive piglets from young or old parity groups.

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

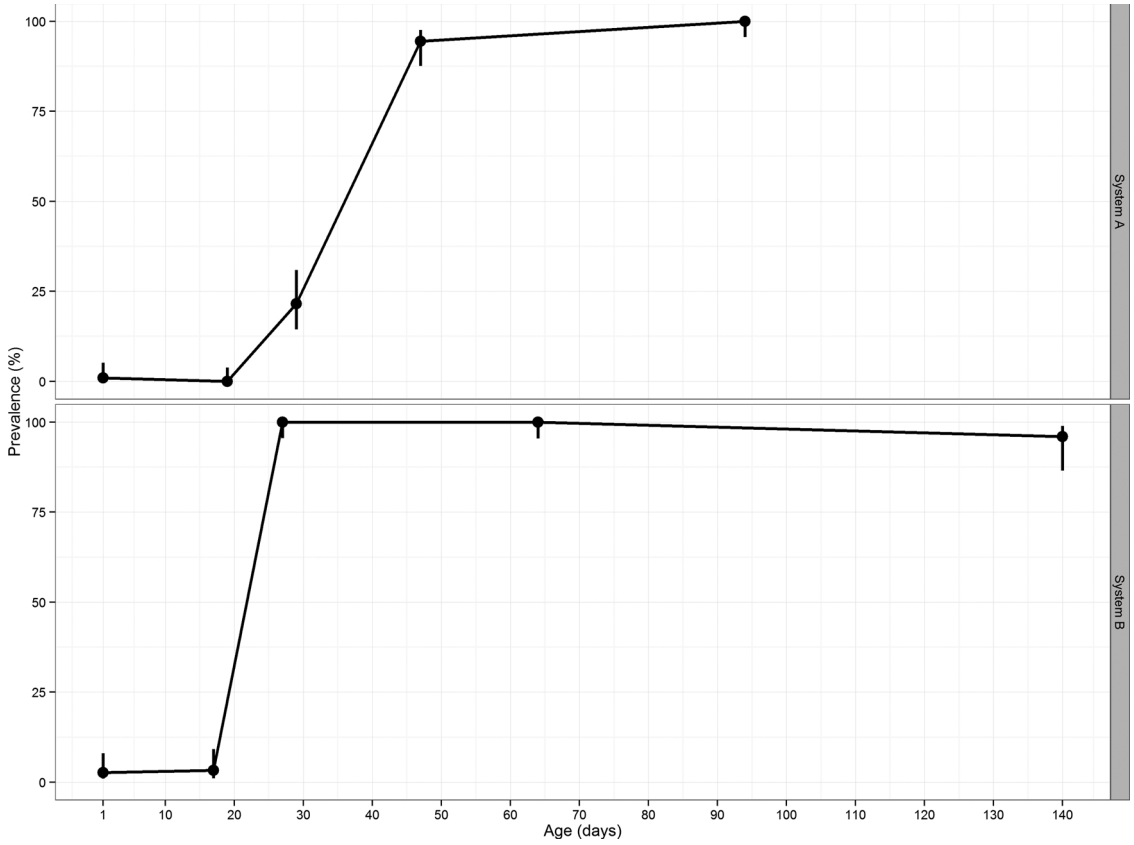

Fig. 1. Prevalence (95% CI) of *M. hyorhinis* infection by pig age in System A (upper) and System B (lower).

3.1.2. Growing pigs

In contrast to the low prevalence observed in piglets until weaning, most pigs became colonized during the first four weeks in the nursery and remained positive throughout the nursery and finishing stages (Fig. 1). Twenty of 93 pigs (22%, 95% CI: 14–31%) sampled in Herd A and all 82 pigs (100%, 95% CI: 96–100%) sampled in Herd B were positive by qPCR at 10 days after placement in the nursery (29 and 27 days of age, respectively).

In each herd, the attending veterinarians determined the timing of the last two sampling events based on clinical signs and mortality data from previous cohorts. For Herd A, 85 of 90 pigs sampled (94% (95% CI: 88%–98%) tested positive by qPCR at 47 days of age, and all 84 (100%, 95% CI: 96–100%) were positive at 94 days of age. In Herd B, all 80 pigs sampled (100%, 95% CI: 94–100%) were positive at 64 days of age, and 48 of 50 (96%, 95% CI: 87–99%) were positive at 140 days of age. The decrease in number of sampled pigs from 80 to 50 at 140 days in Herd B was due to the loss of ear tags. In Herd A, the range of bacterial load was 3.7 to 7.8, 3.6 to 7.8, and 4.3 to 8.3 $\log_{10}$ geq/swab at 29, 47, and 94 days of age, respectively. Similar results were observed in Herd B (Supplemental Fig. 2).

The incidence rate was estimated for the different phases between samplings (Fig. 1). In Herd A, the incidence rate was 2.1 new cases per 100 pig-days (95% CI: 1.3–3.2 new cases per 100 pig-days) from 19 to 29 days of age. The incidence rate increased to 5.1 new cases per 100 pig-days (95% CI: 3.9–6.5) from 29 to 47 days of age and numerically decreased to 1.6 new cases per 100 pig-days (95% CI: 0.5–4.9) at 47 to 94 days of age. In contrast, in Herd B, the incidence rate was 9.4 new cases per 100 pig-days (95% CI: 8.3–12.1) from 17 to 27 days of age, as evidenced by the sharp increase in cases during that period (Fig. 1). Additionally, in system A, the tonsils from a subset of the study pigs (18/20) swabbed at slaughter tested positive, indicating that a pig can remain positive until market age of 200 days.

3.2. Environmental samples

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

3.3. Oral fluids

Detection of *M. hyorhinis* in oral fluid samples was consistent with the detection in the nasal cavity of the study pigs. In both systems, oral fluid samples were collected from post-weaning pigs at three different sampling events. In Herd A, 33% of ropes (3/9, 95% CI: 72–100%) tested positive at 29 days of age (Fig. 2). In subsequent samplings, all oral fluid samples tested positive by qPCR. In Herd B, all oral fluid samples collected from pigs at 27, 64, and 140 days (25/25) were positive by qPCR (Fig. 2). The range of bacterial load from oral fluids collected from Herd A was 4.1 to 5.6 $\log_{10}$ geq/mL, and the range in Herd B was 4.2 to 7.6 $\log_{10}$ geq/mL.

3.4. Serology

Serum samples were collected from 107 and 111 sows and tagged piglets in the farrowing rooms of Herds A and B, respectively. Additionally, serum samples were collected from the same group of pigs at four subsequent sampling events (Supplemental Fig. 1), except in Herd A, where the sampling at weaning was not done. The distributions of serum titers for study pigs are displayed in Fig. 3. A total of 4/107 and 13/111 piglets at birth had no detectable serum antibodies measured by ELISA in Herds A and B, respectively. The median serum titer for piglets at birth was 25,600 and 1600 for systems A and B, respectively (Fig. 3). At weaning, the median titers had decreased to 800 in

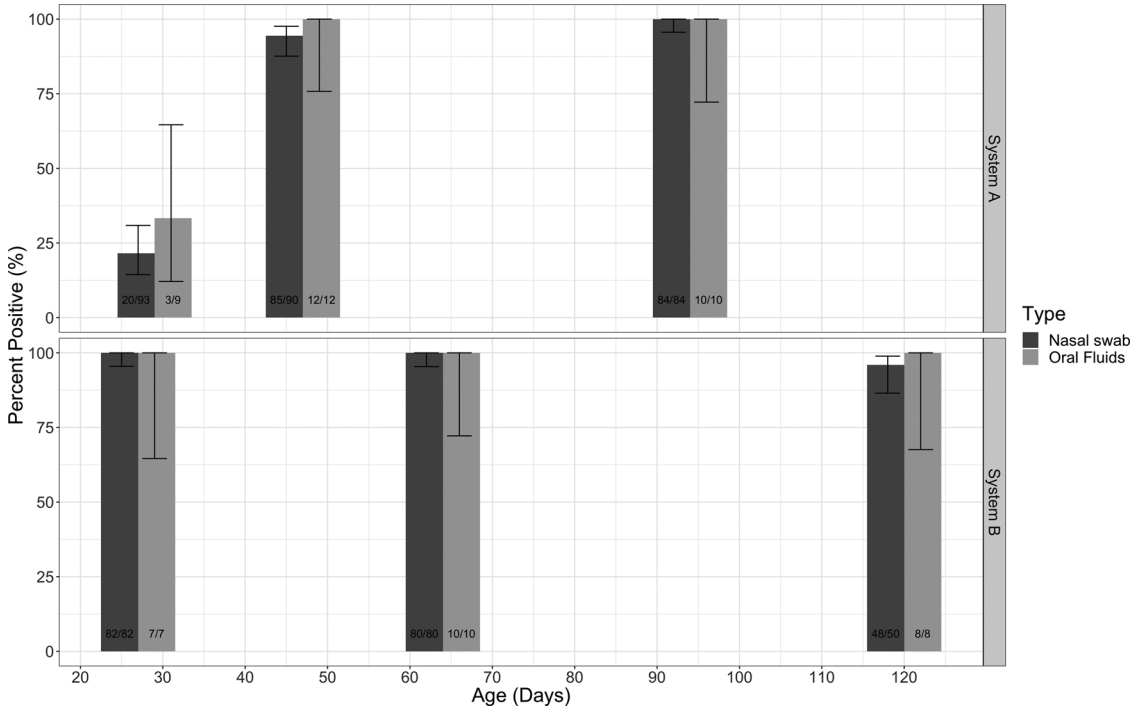

Fig. 2. Proportion of *M. hyorhinis* positive nasal swabs and oral fluid samples in System A (upper) and System B (lower). Error bars represent 95% confidence intervals.

Herd B. After weaning, at the first sampling median serum titers were 6400 in Herd A (29 days post weaning [dpw]) and 800 in Herd B (27 days dpw). Median serum titers were 1600 in both herds at the subsequent sampling at 47 dpw in Herd A and 64 dpw in Herd B. At the final sampling, median titers had increased to 6400 and 3200 in pigs from system A (94 dpw) and B (140 dpw), respectively (Fig. 3). Sow median serum titer values are reported in Table 2.

3.5. Clinical signs and necropsy observations

Typical clinical signs of polyserositis were infrequent during the nursery mortality peak in Herd A, and none of the study pigs showed any clinical signs; therefore, only eight pigs were selected for necropsy from the same cohort of the study pigs. Of those, five pigs had pericarditis. Of these five, two tested positive for *M. hyorhinis* by qPCR of pericardium samples. All pigs were positive for *M. hyorhinis* in the nasal cavity by qPCR. *M. hyorhinis* was cultured from 2/8 bronchial, 2/8 pericardial, and 4/8 nasal swabs. *H. parasuis* was detected in the pericardium of 7/8 by PCR. All three pools of lung tissue were positive for IAV by PCR. Pigs tested negative for PRRSV, *M. hyopneumoniae*, and PCV2. In the grow finishing stage, over 50% of the population had clinical signs of lameness and all 10 necropsied pigs had lesions suggestive of arthritis, such as increased synovial fluid or increased turbidity and hyperemia or edema of joint synovial membranes. Eight samples from joints of these pigs tested positive for *M. hyorhinis* by qPCR, and three were also positive for *Mycoplasma hyosynoviae* by PCR. None of the joint samples tested positive for *S. suis* or *H. parasuis* by PCR. *M. hyorhinis* was also detected by qPCR from all 10 bronchial, pericardial and nasal swabs; it was isolated from all 10 bronchial swabs and two of 10 nasal and pericardial swabs but none of 10 joint swabs. All tissue pools tested positive for IAV and PRRSV. In addition to the eight and 10 sick pigs necropsied during nursery and finishing stages, two healthy pigs were necropsied at each sampling time. *M. hyorhinis* was detected in the nasal cavity of all four healthy pigs and in one of two bronchial swabs from pigs in the finishing. *M. hyorhinis* was not detected, however, in any systemic sites from healthy pigs. The veterinarian of this herd reported that this group of pigs experienced lameness until slaughter age. A total of nine study pigs were sampled at the processing plant. All pigs had swollen joints and 6/9 had lymphoplasmacytic synovitis lesions observed by histopathology. *M hyorhinis* was detected in 9/9 tonsils and 1/9 joint swabs by qPCR. *M. hyosynoviae* was detected in 2/9 tonsils and 1/9 joint swabs by PCR. *S. suis* and *H. parasuis* was not detected in any of the joints.

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

4. Discussion

Genomic analyses indicate that all mycoplasmas have a common gram-positive ancestor, and reductive genome evolution in these organisms has been associated with close adaptation to specific hosts and anatomical niches (Kamminga et al., 2017). Several mycoplasmas (*M. hyopneumoniae, M. hyorhinis, M hyosynoviae, M. suis, M. flocculare*) are host-specific commensals or pathogens of pigs. As in other hosts, however, asymptomatic infections are a hallmark of mycoplasma biology in pigs, including the more pathogenic species. The factors underlying the occurrence and severity of clinical mycoplasmal disease in swine, including *M. hyorhinis,* remain poorly understood. Although long recognized as a common commensal of pigs, there is evidence that clinical *M. hyorhinis* disease may be an emerging problem in intensive swine production (Dos Santos et al., 2015). The goal of this study was to understand the dynamics of colonization and infection with *M. hyorhinis* in clinically affected herds where pigs were raised in multiple site

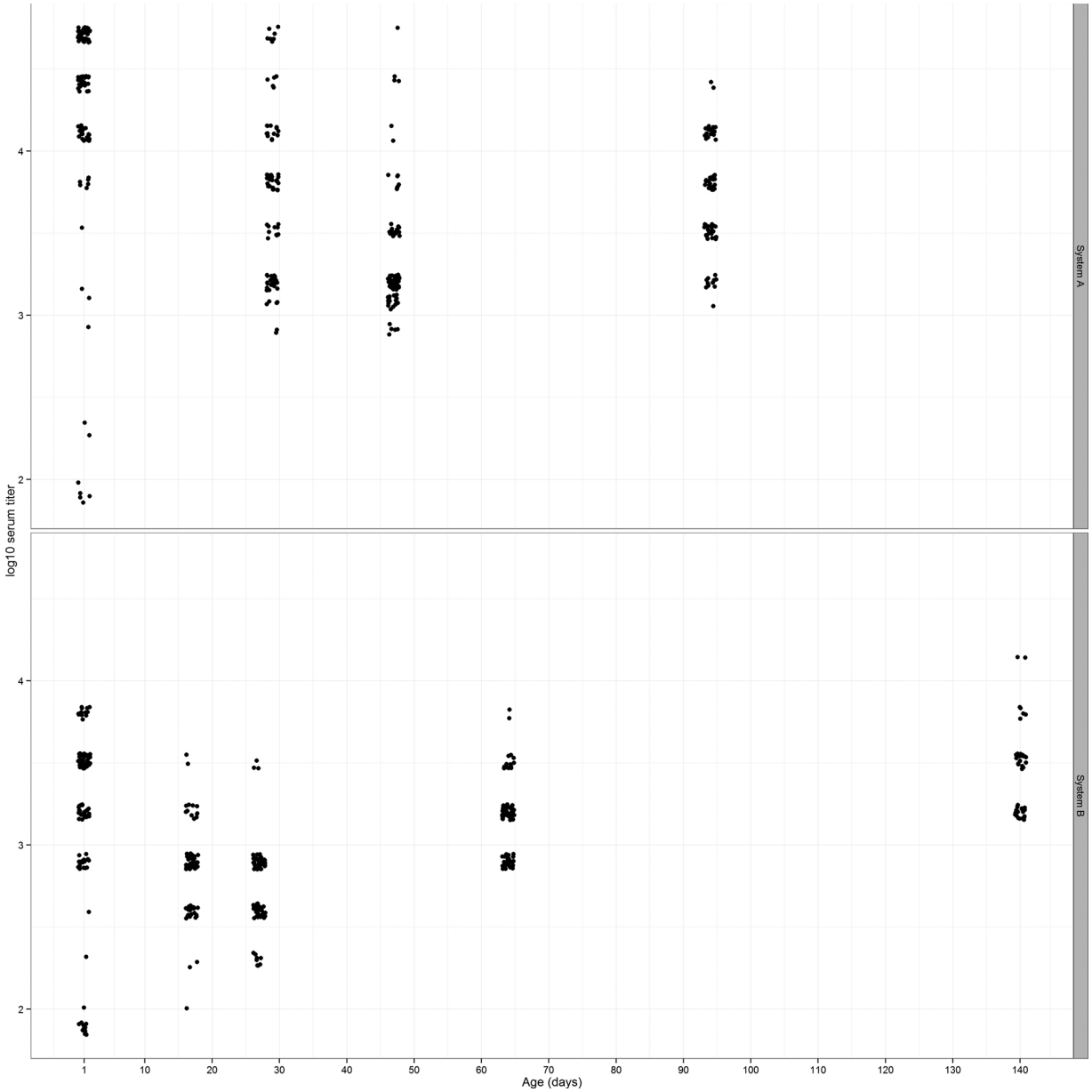

Fig. 3. Serum antibody titers of pigs over time obtained by ELISA in System A (upper) and System B (lower).

systems.

The epidemiology of infectious diseases is substantially influenced by host population demographics and dynamics (Gandon et al., 2016). *M. hyorhinis* is a highly successful bacterium as it appears to be endemic in most swine populations globally, although studies of transmission under natural conditions in wild or feral swine populations are lacking. Over the last three decades, swine production in the United States has undergone substantial transformation, and much of this change was driven by the goal of improving herd health (Davies, 2012). Salient features of these changes are increased herd sizes and multiple site production, of which a key component is rearing of weaned pigs at sites distant from the parent breeding herds. This approach of segregation can affect the epidemiology of infectious agents and may have adverse effects with some diseases such as *Haemophilus parasuis* and *Streptococcus suis* (Oliveira et al., 2001). The apparent emergence of *M. hyorhinis* as a swine pathogen has occurred in an era of widespread adoption of multiple site production in the USA, providing the rationale for focusing the study on such systems.

The observations in this longitudinal study were generally concordant with previous cross-sectional data on prevalence of colonization in breeding and growing pig populations (Clavijo et al., 2017). In a 1973 study, Ross and Spear cultured *M. hyorhinis* from the nasal secretions of 7% and 0.35% of piglets of 6 weeks of age (Ross and Spear, 1973). The low prevalence of *M. hyorhinis* in piglets may be a feature common among *Mycoplasma* species that colonize the mucosa of pigs, including *M. hyopneumoniae* and *M. hyosynoviae*. Hagedorn-Olsen et al. (1999) found between 0–9% of piglets colonized with *M. hyosynoviae* prior to four weeks, and Sibila et al. (2007) found 6.4% of piglets positive for *M. hyopneumoniae* prior to weaning. Fano et al. (2007), however, reported a wide range (0–51%) of *M. hyopneumoniae* prevalence at weaning among 20 cohorts of pigs on three different farms. More investigations are needed to determine if certain management practices can alter the colonization patterns. For example, while systems in the USA commonly wean pigs around 21 days of age, it is possible that systems that wean later (> 28 days) have higher preweaning colonization rates, since they are in longer contact with their sows.

Contrary to a previous study in which younger sows were more

likely to be PCR positive in the nasal cavity, no significant difference in prevalence between parity groups of sows was observed (Clavijo et al., 2017). A study of *M. hyopneumoniae* found no statistical difference in nasal colonization among parities from parity 1 to 7 (Calsamiglia and Pijoan, 2000). In the present study, it is possible that the difference in the proportion of *M. hyorhinis* PCR-positive sows between the young and old groups was smaller in these two herds than in the herds studied in previously (Clavijo et al., 2017), making the estimated sample size insufficient to detect the potential difference, or simply that there is no consistent relationship between parity and *M. hyorhinis* colonization across herds. The identification of the source of infection for suckling piglets is an important component in the understanding of disease epidemiology. Because sows are in close contact with their piglets in the farrowing room, they represent the most likely source of infection for piglets. In this study, however, infected piglets originated from PCR-negative sows, and PCR-positive sows had negative piglets. In addition, no associations were observed between sow parity and seropositivity or with piglet nasal colonization and seropositivity. It is possible that sows shed *M. hyorhinis* intermittently, which has been suggested with *M. hyopneumoniae* (Ruiz et al., 2003) and *Mycoplasma bovis* (Maunsell et al., 2011). Studies of the relationship between the colonization of the sow and the piglet have been carried out for *M. hyopneumoniae,* with contradictory results. Fano et al. (2006) found a higher proportion of positive piglets at weaning originating from young sows (p1-2), while Sibila et al. (2007) found no significant relationships between sow parity and piglet nasal colonization and seropositivity. In another study looking at *M. hyosynoviae* tonsillar carriage in three different swine herds, all sows sampled (n = 12) were colonized in all three herds with *M. hyosynoviae,* while only one herd had 9% (3/34) of piglets colonized at four weeks (Ross and Spear, 1973). Further studies are indicated to clarify the influence of sow parity on *M. hyorhinis* colonization as this could influence the utility of interventions, such as parity segregation, to manage disease observed in the nursery and grow-finish phases. It is also likely that patterns of sow-to-piglet transmission, including parity effects, may vary among farms due to factors such as replacement rate and rearing practices of replacement gilts.

Besides the sow, other possible sources of infection for piglets could be through direct contact with other piglets or indirect contact from contaminated environments or fomites. The indirect route was investigated by testing environmental samples from the farrowing rooms. Negative results for these environmental samples may suggest limited shedding from colonized pigs and/or limited persistence of *M. hyorhinis* outside host animals. Still, it is likely that the low sample size hindered the ability to detect *M. hyorhinis* in the environment, especially under the low-prevalence scenario observed in the farrowing room.

In this study, over 90% of the pigs became colonized during the nursery phase, and the incidence rate estimates confirmed rapid transmission during this stage. This contrasts with much slower transmission reported for *M. hyopneumoniae* (Meyns et al., 2004). In this study, pigs remained positive for most of the post-weaning phase, and a subset of study pigs were positive at slaughter, suggesting prolonged colonization. Prolonged asymptomatic colonization is also a generic feature of Mycoplasma epidemiology, which includes other pathogenic swine mycoplasmas such as *M. hyopneumoniae*, *M. hyosynoviae* (Pieters et al., 2009; Friis et al., 1991), and the hemotrophic species *M. suis* (Hoelzle, 2007).

Although both herds had similar colonization patterns, they had different clinical presentations and timing of *M. hyorhinis* disease. While Herd A had a low proportion of polyserositis cases in the nursery, over 50% of pigs at 94 days of age were clinically lame, and *M. hyorhinis* was detected in all affected joints in necropsied pigs. In contrast, the peak *M. hyorhinis* disease in Herd B occurred in the nursery where pigs presented mainly polyserositis. While the role of viral agents (i.e. PRRSv, IAV and PCV2) in *M. hyorhinis* disease is still unclear, it is interesting to note that in both systems the peak of *M. hyorhinis* disease coincided with detection of IAV and PRRSv in pools of tissue. However, in Herd B, while PRRSV was circulating during the finishing period, *M. hyorhinis* associated disease was not observed. Recent investigations have reported a potential association between *M. hyorhinis* and pneumonia caused by PRRSV (Lee et al., 2016). Still, more research is needed to elucidate the possible potentiation of *M. hyorhinis* disease by viral agents. This information highlights the varying clinical presentations that should be considered when dealing with *M. hyorhinis* disease in the field. Still, the observed infection patterns may not necessarily be the same for successive groups of sows and pigs within a herd. Therefore, research to identify herd-level risk factors for *M. hyorhinis* disease, as well as potential variability within a system between different cohorts, is warranted. Recently, the testing of oral fluids has proven to be a very useful tool for pathogen monitoring and surveillance (Prickett et al., 2008). In this study, the detection of *M. hyorhinis* in oral fluid samples was consistent with its detection in the nasal cavity. Future studies are needed to determine the sensitivity of oral fluids for *M. hyorhinis* detection at the pen level (Ramirez et al., 2012)

In this study, colonized pigs had serum antibodies against *M. hyorhinis*. Since *M. hyorhinis* was detected in the nasal cavity of piglets with colostral-derived antibodies, it appears that maternal antibodies do not completely protect against *M. hyorhinis* nasal colonization. In contrast, a recent study found that passive immunity towards *M. hyosynoviae* seemed to provide partial protection against development of arthritis in piglets (Lauritsen et al., 2017). Shortly after weaning, these antibodies decreased, which coincided with the increase in *M. hyorhinis* prevalence. During the peak of mortality in each system, it appears that there was an increase in serum titers, presumably due to development of acquired immunity. Future studies should evaluate the role of serum antibodies in protection against *M. hyorhinis* disease, as well as the detection of *M. hyorhinis*-specific mucosal antibodies and their impact on infection.

This study provides novel insight on the infection dynamics of *M. hyorhinis* in affected herds. Overall, the prevalence of *M. hyorhinis* infection in sows and piglets was fairly low. In contrast, the prevalence of infection after placement in the nursery was close to 100%, indicating rapid transmission post-weaning. It was also observed that a subset of pigs remained positive until slaughter, suggesting a long bacterial shedding period. Detection in oral fluids were consistent with detection in nasal swabs, potentially making this a suitable, less-invasive and practical sample type to monitor bacterial colonization. This is also the first study to report the pattern of antibody detection in pigs over time. In addition, the different clinical presentations and timing of disease between herds reflect the variation that can be observed in field. Collectively, these findings should be taken into consideration when surveilling pig populations for *M. hyorhinis*. Because of the participation of two different systems, various diagnostic procedures, and the longitudinal nature of this study, the information generated here is relevant and useful to understand the epidemiology of this pathogen in commercial herds. This study provides a framework to understand the dynamics of infection of this pathogen and could aid in the development of control measures to prevent disease in affected herds.

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Conflict of interest statement

The authors declare no conflicts of interest. Maria Clavijo is employed part-time by PIC (Pig Improvement Company).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.05.021.

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