Veterinary Microbiology 241 (2020) 108500

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Comparison of the sensitivity of laryngeal swabs and deep tracheal catheters for detection of *Mycoplasma hyopneumoniae* in experimentally and naturally infected pigs early and late after infection

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ARTICLE INFO

Keywords:
In vivo
Deep tracheal catheter
Laryngeal swab
Sensitivity
Bayesian modeling

ABSTRACT

Detection of *Mycoplasma hyopneumoniae* infection in live pigs is a critical component to measure the success of disease control or elimination strategies. However, *in vivo* diagnosis of *M. hyopneumoniae* is difficult and the imperfect sensitivity of diagnostic tools has been deemed as one of the main challenges. Here, the sensitivity of laryngeal swabs and deep tracheal catheters for detection of *M. hyopneumoniae* early and late after infection was determined using inoculation status as a gold standard in experimentally infected pigs and a Bayesian approach in naturally infected pigs. Three-hundred and twenty 8-week old seeder pigs were intra-tracheally inoculated with *M. hyopneumoniae* strain 232 and immediately placed with 1920 contact pigs to achieve a 1:6 seeder-to-contact ratio. A subset of seeders and contacts were longitudinally sampled at 7, 28, 97, and 113 days post-inoculation (dpi) and at 28, 56, 84, and 113 days post-exposure (dpe), respectively, using laryngeal swabs and deep tracheal catheters. Samples were tested for *M. hyopneumoniae* by a species-specific real-time PCR. The sensitivity of deep tracheal catheters was higher than the one obtained in laryngeal swabs at all samplings (seeders: 36% higher than laryngeal swabs at 7 dpi, 29% higher at 97 dpi, and 44% higher at 113 dpi; contacts: 51% higher at 56 dpe, 42% higher at 84 dpe, and 32% higher at 113 dpe). Our study indicates that deep tracheal catheters were a more sensitive sample than laryngeal swabs. The sensitivity of both sample types varied over time and by exposure method, and these factors should be considered when designing diagnostic strategies.

1. Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is a significant respiratory pathogen of swine worldwide, with involvement in respiratory diseases such as enzootic pneumonia (Mare and Switzer, 1965; Goodwin et al., 1965) and the more severe porcine respiratory disease complex (Dee, 1996). This bacterium is the cause of major economic losses in finishing pigs, due to decreased average daily gain, high feed conversion ratios, and high treatment costs (Maes et al., 2017).

Diagnosis of infection, or lack of, is a critical component to measure the success of disease control or elimination strategies. However, *in vivo* diagnosis of *M. hyopneumoniae* infection is challenging in the acute and chronic stages for several reasons. In experimentally infected pigs, the primary clinical sign associated with acute *M. hyopneumoniae* infection is a dry cough that can initiate between 7 and 14 days post infection (Lorenzo et al., 2006; Fano et al., 2005; Pieters et al., 2009) and cease up to 11 weeks after onset (Kobisch et al., 1993; Sorensen et al., 1997; Fano et al., 2005; Pieters et al., 2009). A large percentage of infected animals then enter the chronic stage of infection, becoming asymptomatic carriers, which can infect susceptible pigs up to 214 days after experimental infection (Pieters et al., 2009). Serologic testing by ELISA is an economical and therefore common method for detection of exposure to *M. hyopneumoniae*. However, ELISAs will not identify experimentally or naturally infected pigs in the early stage of the infection (Sorensen et al., 1993; Sitjar et al., 1996; Kurth et al., 2002). ELISA responses are challenging to interpret in vaccinated populations and there is a potential for cross-reactivity among mycoplasma species

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https://doi.org/10.1016/j.vetmic.2019.108500
Received 19 July 2019; Received in revised form 1 November 2019; Accepted 4 November 2019

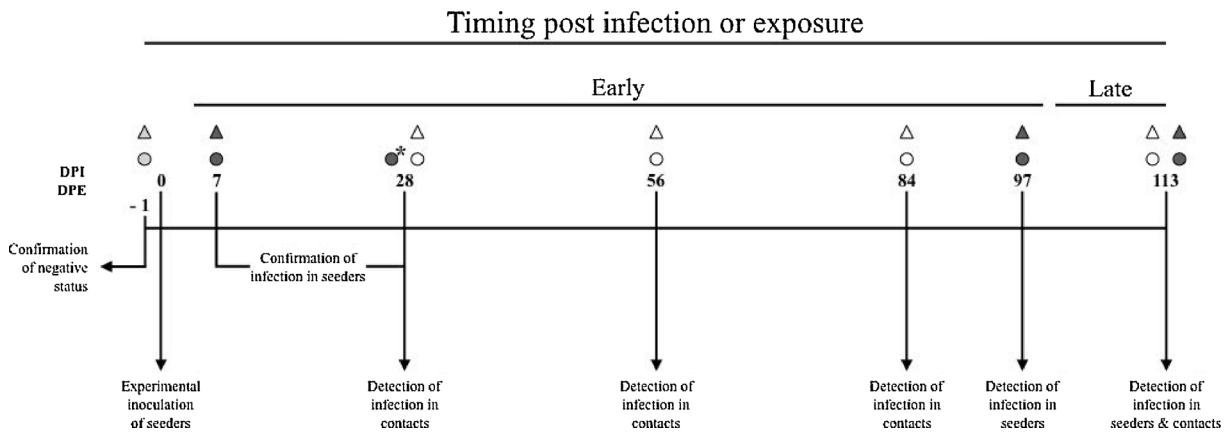

Fig. 1. Graphic representation of the experimental design and timeline. Laryngeal swabs are represented as Δ and deep tracheal catheters are represented as O. Black: Seeders. Blank: Contacts. Light gray: Only a subset of the population. dpi: days post inoculation. dpe: days post exposure. Dpi and dpe are the same days on the timeline. Pigs were approximately 8 weeks of age at 0 dpi/dpe. *(n = 31).

(Freeman et al., 1984; Ameri et al., 2006; Neto et al., 2014; Petersen et al., 2016), which altogether can confound serological diagnosis. Furthermore, even for the same sample, differences in ELISA platforms can lead to variable results and it has been proposed that a combination of assays may assist in diagnosis at the herd level (Erlandson et al., 2005). In comparison, real-time PCR directed to the detection of bacterial DNA allows for a higher degree of accuracy, and is considered the most sensitive test for samples obtained *in vivo* or at necropsy (Maes et al., 2008; Strait et al., 2008).

Colonization of *M. hyopneumoniae* in the lower respiratory tract, where proliferation occurs in greatest numbers (Blanchard et al., 1992; Zielinski and Ross, 1993), complicates *in vivo* diagnostics. Sensitivity of detection *in vivo* is highly dependent on sample type and collection timing. It has been shown that samples obtained from the lower respiratory tract of infected pigs yield higher detection rates than those obtained from the upper respiratory tract (Otagiri et al., 2005; Marois et al., 2007; Fablet et al., 2010; Vangroenweghe et al., 2015; Pieters et al., 2017). Comparisons of the diagnostic performance of different sample types collected during the acute phase of infection following experimental inoculation with *M. hyopneumoniae* are available (Otagiri et al., 2005; Marois et al., 2007; Pieters et al., 2017). Similar studies have been conducted in naturally infected animals, with samples collected during the acute (Vangroenweghe et al., 2015) or chronic phase of infection (Sievers et al., 2015), or with an unknown exposure time point (Otagiri et al., 2005; Fablet et al., 2010). However, to the authors' knowledge no studies comparing the two sample types yielding the highest *in vivo* detection rates, namely laryngeal swabs (Pieters et al., 2017) and deep tracheal catheters (Fablet et al., 2010), collected during both acute and chronic phases of infection with a known exposure time, have been conducted. Furthermore, no studies comparing sample types in seeders (experimentally) and contacts (naturally) infected pigs in a large population under controlled field conditions are available. Therefore, the present study was designed to determine the sensitivity of laryngeal swabs and deep tracheal catheters for *M. hyopneumoniae* detection by real-time PCR in experimentally and naturally infected pigs early and late after infection.

2. Materials and methods

2.1. Ethics statement

All animals participating in this study were cared for following the guidelines of the Institutional Animal Care and Use Committee of Pipestone Applied Research (IACUC #3-18).

2.2. Animal source and housing

Two thousand two hundred and forty crossbred pigs were sourced from a commercial sow farm known to be positive to influenza A virus (IAV-S) and negative to porcine reproductive and respiratory syndrome virus (PRRSv) and *M. hyopneumoniae* infection for the last 5 years, based on clinical observation in the sow herd and downstream flow, and confirmation by repeated diagnostic testing. Diagnostic sample types for detection by PCR consisted of oropharyngeal swabs, oral fluids, and tissue for IAV-S and PRRSv, and deep tracheal catheter and tissue for *M. hyopneumoniae*. Repeated ELISA testing was also performed for *M. hyopneumoniae* antibody detection. At approximately 21 days of age, all pigs were weaned, transported, and housed in a Midwestern United States commercial wean-to-finish facility designed for research, following strict biosecurity measures. Pigs were housed in the research barn for 5 weeks, until the start of the study at 8 weeks of age. The research barn consisted of two rooms with 40 2.4 × 6.1 m pens per room and was representative of a typical Midwestern wean-to-finish tunnel ventilated facility. Pen partitions were horizontal rod (open) gating and all pens had slatted concrete floors. The facility was not filtered and the closest swine facilities were approximately 2.5 km away. The surrounding wean-to-finish facilities were stocked with *M. hyopneumoniae* naïve offspring at weaning. A regular program of surveillance using PCR for detection of *M. hyopneumoniae* by deep tracheal catheters was in place for neighboring sites and a majority of sites within 9.1 km from the research facility. Monitored sites remained *M. hyopneumoniae* negative as determined by PCR and the absence of respiratory clinical signs consistent with *M. hyopneumoniae* as described by Nathues et al. (2012) throughout the course of the study.

2.3. Experimental design

A timeline and graphic representation of the experimental design of this study are shown in Fig. 1. Pigs were allocated into two groups, namely seeders (n = 320) and contacts (n = 1920). Upon arrival to the research barn, both seeder and contact pigs were randomly allocated to one of 80 pens based on a 1:6 seeder-to-contact ratio, for a total of 28 pigs per pen. Fifty seeders (0–1/pen) and 90 contacts (1–2/pen) were randomly selected from the 80 pens, and identified by ear tag for longitudinal sample collection. At 8 weeks of age, a subset of pigs (n = 90) were randomly selected and laryngeal swab and deep tracheal catheter samples were collected to confirm the negative status of the population for *M. hyopneumoniae* using real-time PCR. The following day, seeders were experimentally inoculated with *M. hyopneumoniae* as described below. Contacts were immediately exposed to seeders following inoculation. The longitudinal sample collection occurred from

seeders (n = 50) at 7, 97, and 113 days post-inoculation (dpi), 31 seeders at 28 dpi, and contacts (n = 90) at 28, 56, 84, and 113 days post-exposure (dpe). Sample size for seeders was set considering a total population size of 2240 to detect a within-farm *M. hyopneumoniae* prevalence of 15% assuming a 100% specificity, a level of confidence of 95%, and a 42% sensitivity based on the lowest reported sensitivity for the two sample types (Sievers et al., 2015). Sample size for contacts was calculated similarly using a prevalence of 8%. Seeder sample size at 28 dpe was below the estimated sample size and added as a confirmatory test. All sample size calculations were carried out using the Epitools epidemiological calculator (Sergeant, ESG, 2018). Days post inoculation of seeders and dpe of contacts were concurrent days on the timeline. Pigs were not exposed to antimicrobials known to be effective against *Mycoplasma* species during the study. Individual pigs were treated for clinical signs suggestive of *Streptococcus suis* using Penicillin or Ceftiofur as indicated.

2.4. Experimental inoculation

At 0 dpi, seeders were intra-tracheally inoculated using 10 mL of a lung homogenate containing 1×10^5 CCU/mL of *M. hyopneumoniae* strain 232 (Minion et al., 2004; purchased from Iowa State University, Ames, IA, USA). *Mycoplasma hyopneumoniae* strain 232 is a well referenced strain and is considered of moderate virulence (Fano et al., 2005; Pieters et al., 2009; Neto et al., 2014; Roos et al., 2016; Pieters et al., 2017). The inoculation technique was performed without the use of anesthesia, with the aid of a laryngoscope and mouth gag for visualization, and a catheter (Coviden™ Kendall™ Feeding Tube and Ureal Catheter, Mansfield, MA, USA) for inoculum delivery into the trachea, as previously described by Neto et al. (2014).

2.5. Sample collection

Laryngeal swabs were collected from pigs with the aid of a mouth gag and a laryngoscope. Sterile collection swabs (Puritan® Unitranz-RT® Transport System, Guilford, ME, USA) were inserted in the mouth cavity rotating after they reached the larynx, as described by Pieters et al. (2017). As the study progressed, and pigs grew, 30.5 cm hemostats were used to extend the reach of the swab. Deep tracheal catheters were collected immediately following laryngeal swab collection from the same pigs. A 40 cm catheter was inserted into the trachea as the pig inspired, with the aid of a mouth gag and a laryngoscope. Catheters were rotated up and down, deep into the trachea, as described by Fablet et al. (2010). Immediately after sample collection, the tip of the catheter was inserted into a sterile snap cap tube containing 1 mL of phosphate-buffered saline. The catheter was cut with scissors, leaving the tip in the snap cap tube. The catheter was estimated to reach the tracheo-bronchial bifurcation at all sampling time points based off of inability to move the catheter further during collection and placement of the catheter during routine necropsy of mortalities. All deep tracheal catheters were collected using sterile materials (Coviden™ Kendall™ Feeding Tube and Ureal Catheter, Mansfield, MA, USA; Corning Science Falcon® 5 mL Polystyrene Round-Bottom Tube, Reynosa, Tamaulipas, Mexico). The mouth gag, laryngoscope, hemostats, and scissors were cleaned with disinfecting wipes (Clorox® Disinfecting Wipes; The Clorox Company, Oakland, CA) and sprayed with sterile water between pigs. All laryngeal swab and deep tracheal catheters were refrigerated immediately and submitted to the Health Management Center (HMC; Boehringer Ingelheim Animal Health, Ames, IA) for analysis.

2.6. Sample processing

DNA extraction (MagMAX™Pathogen DNA/RNA Kit and KingFisher™ extraction robot, Life Technologies, Grand Island, NY, USA) and species-specific *M. hyopneumoniae* real-time PCR (VetMAX™ qPCR Master Mix and VetMAX™*M. hyopneumoniae* Reagents Kit, Life Technologies, Grand Island, NY, USA; Roche LightCycler® 480 Roche Life Science, Indianapolis, IN, USA) were performed at the HMC. Samples with a Ct value < 38 were considered positive for *M. hyopneumoniae* based on laboratory internal validation and other veterinary diagnostic laboratories' cutoff values.

2.7. Clinical observation

Pigs were observed at each collection time point for detection of clinical signs, specifically coughing suggestive of *M. hyopneumoniae* infection, defined as a dry and non-productive cough (Nathues et al., 2012). Coughing was evaluated in a group basis (Fano et al., 2005). Early and late infection was defined by the presence or absence of coughing, respectively, in seeders and contacts during the time of observation.

2.8. Data analysis

The number of positive pigs and the Ct value for each sample type for samples collected from seeders and contacts were compared at each sampling using McNemar exact tests and Wilcoxon signed rank tests for paired samples. In all cases, statistical significance was considered when *p*-values were less than 0.05. All comparisons were performed using R (R Development Core Team, 2008).

The sensitivity obtained for each sample type in seeders at each sampling was calculated as the percentage of positive seeders from the total of seeders (challenged animals) using EpiTools along with the Clopper-Pearson (exact) confidence limits (Sergeant, ESG, 2018).

Bayesian modeling was used to estimate the sensitivity of each sample type at each sampling point in contacts since their true status (infected/not infected) could not be determined in the absence of a reference test. Since the test applied to both sample types was based on detection of bacterial DNA (real-time PCR) in two locations of the respiratory tract, their results were assumed to be conditionally dependent (Gardner et al., 2000). Therefore, a two dependent tests – one population model was fitted at each sampling (Branscum et al., 2005). To ensure model identifiability, the analysis incorporated prior information regarding the test sensitivities, while a weakly informative prior was used for the prevalence, and a perfect specificity was assumed as previously reported in the literature (Strait et al., 2008; Fablet et al., 2010). Beta distributions for the sensitivity of each sample type were formulated on BetaBuster (Su, 2019), assuming that the most likely value was the point estimate for the same sample type in seeders, and a 95% confidence that values were above the lowest bound of the 95% confidence interval estimated for seeders (laryngeal swabs = mode 43%, 95% > 15% and deep tracheal catheters = mode 83%, 95% > 57%). A uniform (0,1) distribution was used as the prior for the prevalence. The influence of the priors on the posterior estimates was assessed in a sensitivity analysis consisting in replacing alternatively the informative priors for the sensitivity of each test and the prevalence by uniform (0,1) distributions. The analyses were carried out in WinBUGS (Lunn et al., 2000). Three chains with different starting values were run for 50,000 iterations after burn-out of the initial 5000 iterations and estimates were obtained after thinning the chains by collecting one in 10 consecutive samples to remove autocorrelation. Model convergence was checked visually by assessing the mixing of the chains and more formally using the Gelman-Rubin diagnostic (Gelman and Rubin, 1992).

3. Results

The subset of pigs tested prior to experimental inoculation (-1 dpi) were confirmed negative for *M. hyopneumoniae* using real-time PCR on laryngeal swabs and deep tracheal catheters (data not shown).

Table 1
Diagnostic and clinical data of *seeders*, early and late after infection with *Mycoplasma hyopneumoniae*.

Days post inoculation (dpi)	Diagnostic and clinical data		
	DNA detection[1] # positive/# tested; (%); mean Ct value (± SEM)		Coughing[2]
	Laryngeal swab	Deep tracheal catheter	
7	22/50[a] (44) 34.8[a] (34.3–35.4)	40/50[b] (80) 32.3[b] (31.8–32.8)	+
28		29/31 (94) 30.5 (29.9–31.0)	+
97	28/49[a] (57) 35.1[a] (34.7–35.4)	42/49[b] (86) 31.7[b] (31.2–32.3)	+
113	13/48[a] (27) 34.0[a] (33.3–34.7)	34/48[b] (71) 30.0[b] (29.3–30.7)	–

Different superscript letters within rows denote statistically significant differences (p-value < 0.001).

[1] Real-time PCR. Samples with Ct values < 38 were considered positive.

[2] +: at least one pig coughing in the group at the time of observation. -: no pigs coughing in the group at the time of observation.

3.1. Seeder pigs

Detection of *M. hyopneumoniae* by real-time PCR and clinical data for seeders is presented in Table 1. Real-time PCR positive results were obtained for both sample types (when collected) at all sampling days throughout the study, with deep tracheal catheters yielding a significantly ($p < 0.001$) higher proportion of real-time PCR positive results compared with laryngeal swabs at all samplings (Table 1). The percentage of real-time PCR positive deep tracheal catheters was 36% higher than laryngeal swabs at 7 dpi, 29% higher at 97 dpi, and 44% higher at 113 dpi. Mean Ct values in positive deep tracheal catheters were also significantly ($p < 0.001$) lower than those from positive laryngeal swabs at all samplings. Coughing was observed at 7, 28, and 97 dpi.

Cumulative incidence as determined by deep tracheal catheters reached 100% at 97 dpi compared to 75% at 113 dpi using laryngeal swabs (Fig. 2A).

The mean sensitivity and 95% confidence interval of laryngeal swabs and deep tracheal catheters for *M. hyopneumoniae* detection by real-time PCR at each sampling in seeders is presented in Table 2.

3.2. Contact pigs

Detection of *M. hyopneumoniae* by real-time PCR and clinical data for contact pigs is presented in Table 3. The first real-time PCR positive result was detected at 28 dpe in deep tracheal catheter. Additional positive results in both sample types were obtained at the three subsequent samplings. The percentage of real-time PCR positive deep tracheal catheters was 22% higher than laryngeal swabs at 56 dpe, 40% higher at 84 dpe, and 37% higher at 113 dpe ($p < 0.001$). Similarly, mean Ct values in positive deep tracheal catheters was significantly lower than those obtained from positive laryngeal swabs at 56, 84, and 113 dpe ($p < 0.02$). Coughing was observed at all samplings.

Cumulative incidence as detected by deep tracheal catheters reached 97% at 113 dpe compared to 73% using laryngeal swabs at the same time point (Fig. 2B).

Posterior estimates for the sensitivity obtained using laryngeal swabs and deep tracheal catheters for *M. hyopneumoniae* detection by real-time PCR at each contact sampling are presented in Table 4. The sensitivity obtained using the deep tracheal catheters was always higher

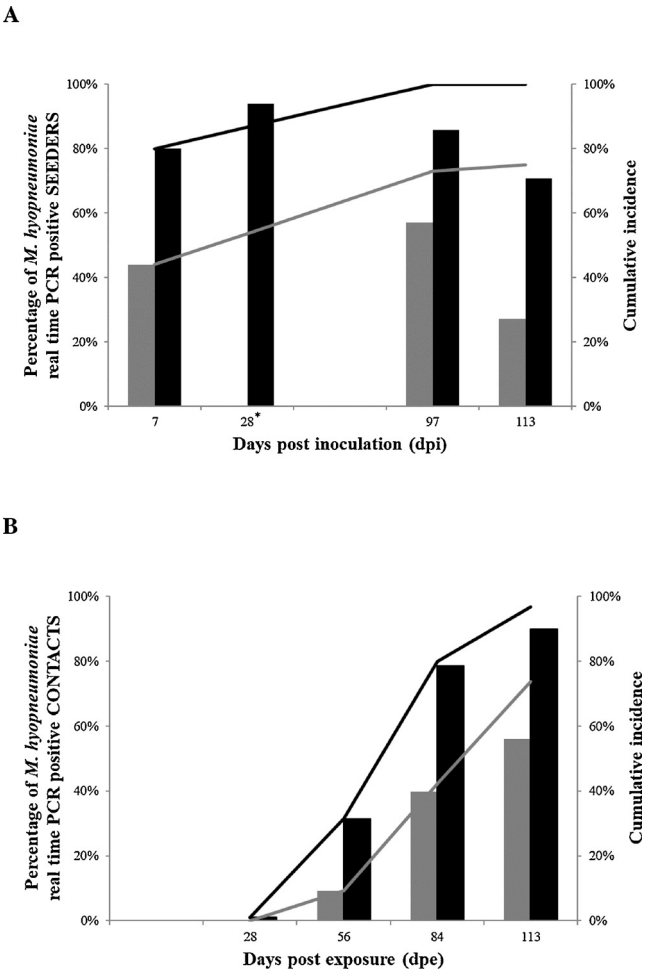

Fig. 2. Prevalence and cumulative incidence of *Mycoplasma hyopneumoniae*. Gray bars: Laryngeal swab prevalence. Black bars: Deep tracheal catheter prevalence. Gray line: Cumulative incidence as detected by laryngeal swabs. Black line: Cumulative incidence as detected by deep tracheal catheters. **A. Seeders**. Samples were obtained during 4 samplings at 7, 28*, 97, and 113 dpi following experimental *M. hyopneumoniae* inoculation. *(n = 31) only DTC collected and no incidence calculated. **B. Contacts**. Samples were obtained during 4 samplings at 28, 56, 84, and 113 dpe following exposure to *M. hyopneumoniae* experimentally inoculated seeders.

than the one obtained in laryngeal swabs at all samplings, especially in the earlier stages of the study (51% higher at 56 dpe, 42% higher at 84 dpe, and 32% higher at 113 dpe). Sensitivity posterior estimates largely agreed with priors except at 56 dpe for the laryngeal swabs (lower posterior estimates) and at 84 and 113 dpe for the deep tracheal catheters (higher posterior estimates; Table 4). Posterior prevalence estimates increased from 0.37 (0.25–0.53 95% PPI) at 56 dpe to 0.86 (0.73–0.99) at 84 dpe and 0.94 (0.86–0.997) at 113 dpe. Conditional dependence between laryngeal swabs and deep tracheal catheters was low although variable depending on the sampling date [0.08 (−0.32–0.37 95% PPI) at 56 dpe, 0.23 (−0.08–0.44) at 84 dpe, and 0.12 (−0.14–0.36) at 113 dpe]. Conditional dependency between test results was assumed in the models given the relatively close proximity between sampling sites, although model results suggested that dependency was in fact low at all sampling dates. Still, using a simpler model without the dependency terms did not lead to change in the posterior estimates (data not shown). No differences in the posterior estimates (changes below 5%) were obtained when the informative priors were replaced with non-informative distributions in the sensitivity analysis (data not shown).

Table 2
Mean and 95% confidence interval of the sensitivity of the two sample types of *seeder Mycoplasma hyopneumoniae* detection by real time PCR.

Days post inoculation (dpi)	Laryngeal swab	Deep tracheal catheter
7	0.44[a] (0.3-0.59)	0.80[b] (0.66-0.9)
28[1]		0.94[b] (0.79-0.99)
97	0.57[a] (0.42-0.71)	0.86[b] (0.73-0.94)
113	0.27[a] (0.15-0.42)	0.71[b] (0.57-0.84)

Clear: early infection. Shaded: late infection. Different superscript letters within rows denote statistically significant differences (p-value < 0.001). [1]31 seeders sampled.

Table 3
Diagnostic and clinical data of *contacts*, early and late after infection with *Mycoplasma hyopneumoniae*.

Days post exposure (dpe)	Diagnostic and clinical data		
	DNA detection[1] # positive/# tested; (%); mean Ct value (± SEM)		Coughing[2]
	Laryngeal swab	Deep tracheal catheter	
28	0/87 (0) 	1/87 (1) 36.7 (36.4–37.1)	+
56	8/86[a] (9) 35.0[a] (34.2–35.8)	27/86[b] (31) 31.4[b] (30.6–32.1)	+
84	33/83[a] (40) 35.4[a] (35.1–35.8)	66/84[b] (79) 29.6[b] (29.3–30.0)	+
113	44/83[a] (53) 34.5[a] (34.1–34.9)	72/80[b] (90) 29.3[b] (28.9–29.7)	+

Different superscript letters within rows denote statistically significant differences (p-value < 0.001).
[1] Real-time PCR. Samples with Ct values < 38 were considered positive.
[2] +: at least one pig coughing in the group at the time of observation. -: no pigs coughing in the group at the time of observation.

4. Discussion

This study was designed to determine the sensitivity of two sample types for *M. hyopneumoniae* detection in experimentally and naturally infected pigs, early and late following infection and/or exposure in a large population under controlled field conditions. In both, experimentally and naturally infected pigs, estimates for the sensitivity of deep tracheal catheters was significantly higher than that obtained for laryngeal swabs. In addition, positive deep tracheal catheters showed significantly lower mean Ct values in seeders and contacts at all samplings compared with positive laryngeal swabs, and the use of deep tracheal catheters also yielded the highest cumulative incidence.

In our study, samples obtained from the lower respiratory tract showed the greatest *M. hyopneumoniae* detection. This is in agreement with other studies (Otagiri et al., 2005; Marois et al., 2007; Fablet et al., 2010; Vangroenweghe et al., 2015) and is consistent with the fact that the trachea and bronchia are considered the multiplication sites of *M. hyopneumoniae* (Blanchard et al., 1992). Samples from the larynx have been proposed as a highly sensitive sample type for real-time PCR detection of *M. hyopneumoniae* during the early stages of infection, under experimental conditions (Pieters et al., 2017). However, detection by laryngeal swabs in seeders at 7 dpi in our study is inconsistent with results obtained by Pieters et al. (2017) who showed 81% of experimentally infected pigs positive at 5 dpi and 86% at 9 dpi. Likewise, Roos et al. (2016) detected 95% and 100% of seeder gilts positive by laryngeal swab at 14 and 28 dpi and 41% and 38% of contacts positive at the same time points. The strain of *M. hyopneumoniae*, inoculation method, inoculation personnel, and laryngeal swab collection technique were similar in all three studies. Nevertheless, the swab type and collection personnel were different. Pieters et al. (2017) and Roos et al. (2016) used a rayon-bud swab, while a nylon-flocked swab was used in the current study. A recent *in vitro* comparison of the two swab types (Takeuti et al., 2017) showed that absorption and detection of *M. hyopneumoniae* was significantly higher in nylon-flocked swabs. It can be hypothesized that the nylon-flocked swab may have become saturated with saliva prior to reaching the larynx in our *in vivo* study, if the tongue of the pig was raised and blocking the pathway to the larynx. Although a similar technique was applied for collection of laryngeal swabs, the variation between sample collectors is believed to be greater for laryngeal swabs *versus* for deep tracheal catheters. In addition, Roos et al. (2016) first exposed contacts to seeders at peak shedding (28 days post inoculation), while in this study contacts were exposed to seeders immediately following inoculation. This may also explain differences observed between detection in contacts in both studies.

The estimated deep tracheal catheter sensitivity in seeders at 113 dpi and contacts at 56 dpe in this study was similar to that reported by Fablet et al. (2010) at an unknown post-exposure period. However, at 113 dpi/dpe the sensitivity of deep tracheal catheter in seeders and of both sample types in contacts were higher than values reported by Sievers et al. (2015), who showed laryngeal samples and deep tracheal catheters to exhibit a similar sensitivity in contacts at approximately 120 dpe. Discrepancies between the two studies could be due to several factors. Most notably, Sievers et al. (2015) used a long handled spoon to collect a mucus sample from the larynx that was then transposed onto a nylon-flocked swab. Furthermore, Sievers et al. (2015) collected deep tracheal catheters prior to the collection of laryngeal samples, which could have inadvertently increased the sensitivity of laryngeal samples. Johnson et al. (2018) detection at 200 and 215 days of age at an unknown exposure time point was lower than detected by laryngeal swabs and deep tracheal catheters in seeders and contacts at 113 dpi and dpe in our study. Variation in exposure timing, timing of collection, and sample collection technique could explain these differences.

The detection pattern of seeders in deep tracheal catheters rose sharply at 7 dpi, peaked at 28 dpi, and gradually declined through 113

Table 4
Mean and 95% posterior probability interval of the estimates for the sensitivity of the two sample types of *contact Mycoplasma hyopneumoniae* detection by real time PCR and the estimates for prevalence, according to a latent class model.

Days post exposure (dpe)	Laryngeal swab	Deep tracheal catheter	Prevalence
56	0.30 (0.16-0.47)	0.85 (0.64-0.97)	0.37 (0.25-0.53)
84	0.46 (0.34-0.58)	0.89 (0.76-0.98)	0.86 (0.73-0.99)
113	0.59 (0.48-0.69)	0.91 (0.83-0.97)	0.94 (0.86-0.997)

Clear: early infection. Shaded: late infection.

dpi. On the other hand, the detection pattern of contacts lagged behind seeders and rose gradually. Neither a decline in the detection pattern of contacts or cessation of coughing amongst contacts was observed prior to the end of the study. Therefore, acute and chronic phases of infection were not clearly differentiated. Instead, the sensitivity of seeders and contatcs prior to and after 100 dpi and dpe were classified as early and late infection, respectively, based on Fano et al. (2005), where coughing in seeders and direct contacts ceased between 63 and 91 dpi/dpe and coughing in indirect contacts ceased between 119 and 155 dpe.

The high proportion of contact pigs detected by real-time PCR suggest that the 1:6 seeder-to-contact ratio used here was highly successful to ensure transmission of *M. hyopneumoniae* over the 16-week period of study. Transmission to almost all contact pigs (97%), suggests that the transmission rate was equal or higher than previous estimates of 1 new infection per infectious pig over a 6-week period (Meyns et al., 2004) and perhaps lower than previous estimates of 3.08 new infections per infectious pig over a 28-day period (Roos et al., 2016). Discrepancies from the later study could be explained by Roos et al. (2016) first exposing contacts to seeders at peak shedding (28 days post inoculation), while in this study contacts were exposed to seeders immediately following inoculation and Meyns et al. (2014) exposed 2 days after inoculation. Still, additional analyses would be needed to quantify the transmission rate in this study.

Mycoplasma hyopneumoniae collected from air samples has been shown to be infectious at distances up to 9.1 km (Otake et al., 2010). Lateral transmission of *M. hyopneumoniae* from a positive population within a 9.1 km radius cannot be definitively ruled out in this study. However, based on the knowledge of the swine populations in neighboring facilities, lateral transmission was assumed to be of low risk.

This investigation incorporated the infection of only one strain of *M. hyopneumoniae*. It is important to consider that various strains of *M. hyopneumoniae* circulate in the field and those may have behave differently based on their virulence (Vicca et al., 2003; Meyns et al., 2007) which can affect the colonization process and detection dynamics. Moreover, different strains may have improved capabilities to colonize some areas of the respiratory tract. In the porcine respiratory disease complex, *M. hyopneumoniae* interacts with other respiratory pathogens, such as PRRSv and IAV-S (Dee, 1996), which we hypothesize may influence the bacterial load in the airways and the dynamics of infection. In the present study, the population was confirmed IAV-S positive and wild-type PRRSv negative using PCR on oral fluids. Assessment of the effect of co-infections on the detection of *M. hyopneumoniae* in the different parts of the respiratory tract is still necessary.

Results of this study provide sensitivities for the two sample types yielding the highest *in vivo* detection rates for *M. hyopneumoniae* by real-time PCR in experimentally and naturally infected pigs in both early and late infection under controlled field conditions. Only two previous studies have reported sensitivities for one or both of the sample types evaluated in this investigation (Fablet et al., 2010; Sievers et al., 2015), and neither of these studies assessed potential sensitivity variations in either phase of infection or exposure method. Bayesian analysis has previously been used to estimate the sensitivity of deep tracheal catheters for detection of *M. hyopneumoniae* infection in a natural contact scenario (Fablet et al., 2010). However, no studies have used either sample type with a known exposure time point over an extended period of time. The sensitivity of the sampling methods was suspected to vary over the course of infection (Moorkamp et al., 2008; Fablet et al., 2010) and exposure method should affect colonization dynamics. Assessment of the effect of the stage of infection on the sensitivity of *M. hyopneumoniae* by the two sample types was necessary to determine more accurate sample size estimates for *M. hyopneumoniae*, tailored to specific scenarios. In this study, sensitivities from seeders were used as prior estimation as the sample technique was the same for both sample types, and the seeders were considered a separate, yet comparable, population in spite of the different exposure method. Regardless, the sensitivity analysis demonstrated that priors did not influence posterior estimates, thus further supporting the robustness of our results.

It is important to note that in this study we determined the sensitivity of individual samples and did not evaluate the effect of sample aggregation. Further studies could be directed towards estimating the sensitivity of laryngeal swabs and deep tracheal catheters for detection of *M. hyopneumoniae* when samples are pooled. Estimating the effect of pooling on sensitivity would be important in low prevalence scenarios, for the development of low cost sampling protocols to be applied in commercial settings without significantly compromising the likelihood of detection.

In conclusion, the sensitivity of *M. hyopneumoniae* detection *in vivo* was highly dependent on sample type and collection timing. These results indicate that deep tracheal catheters were the most sensitive method for detection of *M. hyopneumoniae* in experimentally and naturally infected live pigs using real-time PCR. Furthermore, the sensitivity of deep tracheal catheters changed over the course of infection indicating that one sampling time point may not be enough for confirmation of the entire population exposure.

Declaration of Competing Interest

The authors state no conflict of interest. Boehringer Ingelheim Animal Health USA Inc. provided funding for the study and employs Drs. Sponheim, Fano, and Wetzell and Ethan Schmalling. However, commercial products of the sponsoring company were not evaluated in this study.

Acknowledgments

The authors would like to thank the personnel from Pipestone Applied Research and Boehringer Ingelheim Animal Health USA Inc. who helped with sample collection. Financial support for this study was provided by Boehringer Ingelheim Animal Health USA Inc.

References

Ameri, M., Zhou, E.M., Hsu, W.H., 2006. Western blot immunoassay as a confirmatory test for the presence of anti-*Mycoplasma hyopneumoniae* antibodies in swine serum. J. Vet. Diagn. Invest. 18, 198–201.

Blanchard, B., Vena, M., Cavalier, A., Le Lannic, J., Gouranton, J., Kobisch, M., 1992. Eclectron microscopic observation of the respiratory tract of SPF piglets inoculated with *Mycoplasma hyopneumoniae*. Vet. Microbiol. 30, 329–341.

Branscum, A., Gardner, I., Johnson, W., 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. Prev. Vet. Med. 68 (2–4), 145–163.

Dee, S., 1996. The porcine respiratory disease complex: are subpopulations important? J. Swine Health Prod. 4, 147–149 (Commentary).

Erlandson, K.R., Evans, R.B., Thacker, B.J., Wegner, M., Thacker, E.L., 2005. Evaluation of three serum antibody enzyme-linked immunosorbent assays for *Mycoplasma hyopneumoniae*. J. Swine Health Prod. 13, 198–203.

Fano, E., Pijoan, C., Dee, S., 2005. Dynamics and persistence of *Mycoplasma hyopneumoniae* infection in pigs. Can. J. Vet. Res. 69, 223–228.

Fablet, C., Marois, C., Kobisch, M., Madec, F., Rose, N., 2010. Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach. Vet. Microbiol. 143, 238–245.

Freeman, M.J., Armstrong, C.H., Sands-Freeman, L.L., Lopez-Osuna, M., 1984. Serological cross-reactivity of porcine reference antisera to *Mycoplasma hyopneumoniae, M. flocculare, M. hyorhinis* and *M. hyosynoviae* indicated by the enzyme-linked immunosorbent assay complement fixation and indirect hemagglutination tests. Can. J. Comp. Med. 48, 2002–2207.

Gardner, I.A., Stryhn, H., Lind, P., Collins, M.T., 2000. Conditional dependence between tests affects the diagnosis and surveillance of animal diseases. Prev. Vet. Med. 45, 107–122.

Gelman, A., Rubin, D.B., 1992. Inference from iterative simulation using multiple sequences. Stat. Sci. 7, 457–511.

Goodwin, R.F., Pomeroy, A.P., Whittlestone, P., 1965. Production of enzootic pneumonia in pigs with mycoplasma. Vet. Rec. 77, 1247–1249.

Johnson, C., Farkas, A., Cano, J.P., Clavijo, M.J., 2018. What happens when *M. hyopneumoniae* enters a herd? Longitudinal assessment of *M. hyopneumoniae* natural infection in gilts. ESPHM 10th European Symposium of Porcine Health Management, Barcelona, Spain. pp. 84.

Kobisch, M., Blanchard, B., Le Potier, M.F., 1993. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and resistance to reinfection. Vet. Res. 24, 67–77.

Kurth, K.T., Hsu, T., Snook, E.R., Thacker, E.L., Thacker, B.J., Minion, F.C., 2002. Use of a

Mycoplasma hyopneumoniae nested polymerase chain reaction test to determine the optimal sampling sites in swine. J. Vet. Diagn. Invest. 14, 463–469.
Lorenzo, H., Quesada, O., Assuncao, P., Castro, A., Rodriguez, F., 2006. Cytokine expression in procine lungs experimentally infected with Mycoplasma hyopneumoniae. Vet. Immunol. Immunopathol. 109, 199–207.
Lunn, D., Thomas, A., Spiegelhalter, D., 2000. WinBUGS – a Bayesian modeling framework: concepts, structure and extensibility. Stat. Comput. 10, 325–337.
Maes, D., Segales, J., Meyns, T., Sibila, M., Pieters, M., Haesebrouck, F., 2008. Control of Mycoplasma hyopneumoniae infections in pigs. Vet. Microbiol. 126, 297–309.
Maes, D., Sibila, M., Kuhnert, P., Segales, J., Haesebrouck, F., Pieters, M., 2017. Update on *Mycoplasma hyopneumoniae* infections in pigs: knowledge gaps for improved disease control. Transbound. Emerg. Dis. 1–15.
Mare, C.J., Switzer, W.P., 1965. New Species: *Mycoplasma hyopneumoniae*; a causative agent of virus pig pneumonia. Vet. Med. Small Anim. Clin. 60, 841–846.
Marois, C., Le Carrou, J., Kobisch, M., Gautier-Bouchardon, A.V., 2007. Isolation of *Mycoplasma hyopneumoniae* from different sampling sites in experimentally infected and contact SPF piglets. Vet. Microbiol. 120, 96–104.
Meyns, T., Maes, D., Dewulf, J., Vicca, J., Haesebrouck, F., de Kruif, A., 2004. Quantification of the spread of *Mycoplasma hyopneumoniae* in nursery pigs using transmission experiments. Prev. Vet. Med. 66, 265–275.
Meyns, T., Maies, D., Calus, D., Ribbens, S., Dewulf, J., Chiers, K., de Kruif, A., Cox, E., Decostere, A., Haesebrouck, F., 2007. Interactions of highly and low virulent *Mycoplasma hyopneumoniae* isolates with the respiratory tract of pigs. Vet. Microbiol. 120 97-95.
Minion, F.C., Lefkowitz, E.J., Madsen, M.L., Cleary, B.J., Swartzell, S.M., Mahairas, G.G., 2004. The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. J. Bacteriol. 186, 7123–7133.
Moorkamp, L., Nathues, H., Spergser, J., Tegeler, R., Beilage, Eg., 2008. Detection of respiratory pathogens in porcine lung tissue and lavage fluid. Vet. J. 175, 273–275.
Nathues, H., Spergser, J., Rosengargen, R., Kreienbrock, L., Beilage, Eg., 2012. Value of the clinical examination in diagnosing enzootic pneumonia in fattening pigs. Vet. J. 193, 443–447.
Neto, J.C.G., Strait, E.L., Raymond, M., Ramirez, A., Minion, F.C., 2014. Antibody responses of swine following infection with *Mycoplasma hyopneumoniae, M. hyorhinis, M. hyosynoviae* and *M. flocculare*. Vet. Microbiol. 174, 163–171.
Otagiri, Y., Asai, T., Okadam, M., Uto, T., Yazawa, S., Hirai, H., Shibata, I., Sato, S., 2005. Detection of *Mycoplasma hyopneumoniae* in lung and nasal swab samples from pigs by nested PCR and culture methods. J. Vet. Med. Sci. 67, 801–805.
Otake, S., Dee, S., Corzo, C., Oliveira, S., Deen, J., 2010. Long-distance airborne transport of infectious PRRSV and *Mycoplasma hyopneumoniae* from a swine population infected with multiple viral variants. Vet. Microbiol. 145, 198–208.
Petersen, A.C., Oneal, D.C., Seibel, J.R., Poel, K., Daum, C.L., Djordjevic, S.P., Minion, F.C., 2016. Cross reactivity among the swine mycoplasmas as identified by protein microarray. Vet. Microbiol. 192, 204–212.
Pieters, M., Pijoan, C., Fano, E., Dee, S., 2009. An assessment of the duration of *Mycoplasma hyopneumoniae* infection in an experimentally infected population of pigs. Vet. Microbiol. 134, 261–266.
Pieters, M., Daniels, J., Rovira, A., 2017. Comparison of sample types and diagnostic methods for in vivo detection of *Mycoplasma hyopneumoniae* during early stages of infection. Vet. Microbiol. 203, 103–109.
R Development Core Team, 2008. R: a Language and Environment for Statistical Computing [Computer Software]. R Foundation for Statistical Computing. Vienna, Austria. Available from. http://www.r-project.org/.
Roos, L.R., Fano, E., Homwong, N., Payne, B., Pieters, M., 2016. A model to investigate the optimal seeder-to-naïve ratio for successful natural *Mycoplasma hyopneumoniae* gilt exposure prior to entering the breeding herd. Vet. Microbiol. 184, 51–58.
Sergeant, ESG, 2018. Epitools Epidemiological Calculators. Available from:. Ausvet Pty Ltd.. http://epitools.ausvet.com.au.
Sievers, C., Dalquist, L., Sponheim, A., Fano, E., Wetzell, T., 2015. Comparison of antemortem sampling procedures to post-mortem bronchial sampling for the detection of *Mycoplasma hyopneumoniae* by PCR. In: Proceedings of the American Association of Swine Veterinarians Meeting. Orlando, FL. pp. 115–116.
Sitjar, M., Noyes, E.P., Simon, X., Pijoan, C., 1996. Relationships among seroconversion to *Mycoplasma hyopneumoniae*, lung lesions, and production parameters in pigs. Swine Health Prod. 4, 273–277.
Sorensen, V., Barfod, K., Feld, N.C., Vraa-Andersen, L., 1993. Application of enzyme-linked immunosorbent assay for the surveillance of *Mycoplasma hyopneumoniae* infection in pigs. Rev. Sci. Technol. 12, 593–604.
Sorensen, V., Ahrens, P., Barfod, K., Feenstra, A.A., Feld, N.C., Friis, N.F., Bille-Hansen, V., Jensen, N.E., Pedersen, M.W., 1997. *Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and evaluation of four diagnostic assays. Vet. Microbiol. 54, 23–34.
Strait, E.L., Madsen, M.L., Minion, F.C., Christopher-Hennings, J., Dammen, M., Jones, K.R., Thacker, E.L., 2008. Real-time PCR assays to address genetic diversity among strains of Mycoplasma hyopneumoniae. J. Clin. Microbiol. 37, 620–627.
Su, C.-L., 2019. BetaBuster. Available from:. https://cadms.vetmed.ucdavis.edu/diagnostic/software.
Takeuti, K.L., de Barcellos, D.E.S.N., Pieters, M., 2017. *Mycoplasma hyopneumoniae* detection in nylon-flocked and rayon-bud swabs. J. Mic. Meth. 141, 118–120.
Vangroenweghe, Frédéric, et al., 2015. Assessment of litter prevalence of *Mycoplasma hyopneumoniae* in preweaned piglets utilizing an antemortem tracheobronchial mucus collection technique and a real-time polymerase chain reaction assay. J. Vet. Diagn. Invest. 27 (5), 606–610.
Vicca, J., Stakenorg, T., Maes, D., Butaye, P., Peeters, J., de Kruif, A., Haesebrouck, F., 2003. Evaluation of virulence of *Mycoplasma hyopneumoniae* field isolates. Vet. Microbiol. 97, 177–190.
Zielinski, G.C., Ross, R.F., 1993. Adherence of *Mycoplasma hyopneumoniae* to porcine ciliated respiratory tract cells. Am. J. Vet. Res. 54, 1262–1269.