



## A diagnostic approach to confirm *Mycoplasma hyopneumoniae* “Day zero” for pathogen eradication

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### ABSTRACT

Breeding herds in the US are trending toward eradication of *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) due to the positive impact on welfare and downstream production. In an eradication program, “Day 0” is the time point when the last replacement gilts to enter the herd were exposed to *M. hyopneumoniae* and marks the beginning of a herd closure. However, no specific diagnostic protocols are available for confirmation of successful exposure to define Day 0. Therefore, the objective of this study was to develop diagnostic guidelines, including sample collection approaches, for two common gilt exposure methods to confirm an entire population has been infected with *M. hyopneumoniae* following purposeful exposure. Forty gilts, age 21–56 days, were ear-tagged for longitudinal sample collection at five commercial gilt developer units (GDUs) and were exposed to *M. hyopneumoniae* by natural contact or aerosolization. Study gilts originated from sources known to be negative to major swine pathogens, including *M. hyopneumoniae*, and were sampled prior to exposure to confirm negative status, then every two weeks. Blood samples were collected for antibody detection, while laryngeal and deep tracheal secretions and pen based oral fluids were collected for PCR, and sampling continued until at least 85% of samples were positive by PCR. Detection of *M. hyopneumoniae* varied greatly based on sample type. Oral fluids showed the lowest detection in groups of gilts detected positive by other sample types. Detection by PCR in deep tracheal secretions was higher than in laryngeal secretions. Seroconversion to and PCR detection of *M. hyopneumoniae* on oral fluids were delayed compared to PCR detection at the individual level. By two weeks post-exposure, at least 85% of study gilts in three GDUs exposed by aerosolization were PCR positive in deep tracheal secretions. Natural contact exposure resulted in 22.5% of study gilts becoming PCR positive by two weeks post-initial exposure, 61.5% at four weeks, and 100% at six weeks on deep tracheal secretions. Deep tracheal secretions required the lowest number of gilts to sample for the earliest detection compared to all other samples evaluated. As observed in one of the GDUs using aerosolization, demonstration of failure to expose gilts to *M. hyopneumoniae* allowed for early intervention in the exposure protocol and delay of Day 0, for accurate timing of the eradication protocol. Sampling guidelines proposed in this study can be used for verification of *M. hyopneumoniae* infection of gilts following exposure to determine Day 0 of herd closure.

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## 1. Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is a highly prevalent and economically important swine respiratory pathogen, causing significant disease burden and economic losses due to increased medication use and decreased pig performance (Pieters and Maes, 2019). The economic impact of *M. hyopneumoniae* infections on downstream production (Dykhuis Haden et al., 2012; Silva et al., 2019) has led to the pursuit of control or elimination of the pathogen from swine populations globally. The combination of herd closure, medication, and vaccination is a common approach for *M. hyopneumoniae* eradication in North America (Holst et al., 2015). One of the most important components of a herd closure is the determination of Day 0, which is the time point at which the last replacement gilts entering the herd were exposed to *M. hyopneumoniae*, and marks the beginning of the closure (Yeske et al., 2020). The two most common methods for gilt *M. hyopneumoniae* exposure in the North American swine industry include a seeder model with natural transmission to contacts (Roos et al., 2016) and aerosolization of lung tissue containing the bacterium (Toohill, 2017; Yeske, 2017; Robbins et al., 2019). Despite the importance of Day 0 determination with either exposure method, diagnostic confirmation of successful replacement gilt exposure is challenging, and often overlooked.

Antibody detection via ELISA testing is a commonly used, inexpensive method for *M. hyopneumoniae* herd surveillance in the field, although early-stage infection cannot be detected with this assay (Sorensen et al., 1993; Sitjar et al., 1996; Kurth et al., 2002; Sibila et al., 2009; Pieters et al., 2017; Betlach et al., 2020). Moreover, infection and vaccination responses are indistinguishable (Garcia-Morante et al., 2022). Antigen detection by PCR allows for identification of infected pigs prior to the observation of clinical signs (Pieters et al., 2017), although at an added diagnostic cost when compared to ELISA. PCR sample types from the lower airways such as laryngeal (Pieters et al., 2017) and deep tracheal secretions (Fablet et al., 2010; Vangroenweghe et al., 2015; Sponheim et al., 2020) have increased the sensitivity of detection in live pigs compared to upper airways samples and are an individual sample type allowing for an estimation of prevalence versus aggregated samples.

Given the need for confirmation of successful exposure of replacement gilts for determination of Day 0, the following study was designed to determine the appropriate timing of sample collection and testing approach for detection of *M. hyopneumoniae* post-exposure after two commonly applied methods of gilt exposure. An additional objective was to develop sample collection schemes, at various population sizes, population sensitivity and specificities, and prevalence levels, to confirm population *M. hyopneumoniae* infection following purposeful exposure.

## 2. Materials and methods

### 2.1. Ethics statement

Pigs in this study were under veterinary oversight and care, with a veterinarian-client-patient relationship and a Welfare Quality based certification in place. Additionally, all pigs sampled as part of this study were cared for following the guidelines of the Institutional Animal Care and Use Committee of Boehringer Ingelheim Animal Health Inc.

### 2.2. Description of herds and facilities

Five commercial gilt developer units (GDUs), each belonging to separate sow farms located in the Midwestern United States and using natural contact or aerosolization for *M. hyopneumoniae* exposure, were enrolled in the study. All farms implemented standard biosecurity measures, such as downtime requirement, shower-in and out, clothing, and footwear change. Farms were selected based on a positive status to *M. hyopneumoniae* measured by ELISA, with a history of respiratory clinical signs, and the purchase of negative replacement gilts housed in

separate facilities from the sow farm. A summary of study gilt facilities, gilt flow, age, number of pens, and gilts included in this study is shown in Table 1. Study gilts originated from sources known to be negative to major swine pathogens, including *M. hyopneumoniae* and porcine reproductive and respiratory syndrome virus (PRRSV), as determined by a history of freedom of the pathogen for several years, routine diagnostics, and absence of clinical signs. Study gilts were housed together with non-study gilts in pens, were not vaccinated for *M. hyopneumoniae* and were to remain unvaccinated for the bacterium for the duration of the study. Study gilts and their pen mates were allowed to be treated with antibiotics that were considered to not have activity against *M. hyopneumoniae*, if needed.

### 2.3. Timeline and sample collection

Approximately zero to three days prior to the start of the study, *M. hyopneumoniae* naïve gilts arrived at each of five commercial GDUs and were placed in pens. Forty gilts at each GDU were randomly selected and individually ear tagged for identification on study day zero. Sample size was set considering a total population size of 1000, to detect a within-farm *M. hyopneumoniae* prevalence of at least 20% with a confidence level of 95%, assuming a 100% specificity and a 42% sensitivity (Sievers et al., 2015) based on the lowest reported sensitivity estimates for the two individual PCR sample types considered here (see below) and allowing four extra individuals for loss of follow up over the course of sampling (n = 36). A summary of sample types and collection days for each exposure method is shown in Table 2. Sampling on 0 days post-exposure (dpe) occurred immediately prior to exposure at each GDU in order to confirm negative status.

### 2.4. Exposure method

#### 2.4.1. Natural contact

This method was used in one GDU as follows: three seeder gilts were added to each pen of 29 naïve gilts. Seeder gilts were approximately 130 days of age, previously exposed to *M. hyopneumoniae* on-site at 56 days of age via natural contact and confirmed PCR positive by laryngeal secretions on day zero of the study. Seeder gilts remained in the same pens for the duration of the study.

#### 2.4.2. Aerosolization

Four GDUs in the study used aerosolization of lung tissue containing *M. hyopneumoniae* as an exposure method (Toohill, 2017; Yeske, 2017; Robbins et al., 2019; Figueras Gourgues et al., 2020). Gilts were exposed via aerosolization of a lung homogenate originated from gilts of the same farm as described by Robbins et al. (2019) and Figueras Gourgues et al. (2020).

Laryngeal and deep tracheal secretions, blood samples, and oral fluids were collected at various time points, as shown in Table 2. Laryngeal secretions and oral fluid samples were not collected at Aerosolization GDUs 3 or 4. Laryngeal secretions were collected from gilts as described by Pieters et al. (2017) with the aid of a mouth speculum and laryngoscope. All swabs were collected using sterile collection swabs (Puritan® Unitranz-RT® Transport System, Guilford, ME, USA). Deep tracheal secretions were collected immediately following laryngeal secretion collection from the same gilts, 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. All deep tracheal secretions were collected using sterile materials (Covidien™ Kendall™ Feeding Tube and Ureal Catheter, Mansfield, MA, USA; Corning Science Falcon® 5 mL Polystyrene Round-Bottom Tube, Reynosa, Tamaulipas, Mexico). The mouth speculums, laryngoscope, hemostats, and scissors were cleaned with disinfecting wipes (Clorox® Disinfecting Wipes; The Clorox Company, Oakland, CA) and sprayed with sterile water between pigs to be

**Table 1**  
Summary of gilt developer unit (GDU) background and management.

| Exposure method      | GDU facility location | GDU gilt flow                                    | GDU study room population size | Number of seeder gilts per pen | Number of foggers per study room | Age at exposure (days) | Number of pens sampled | Number of gilts sampled out of total number per pen |
|----------------------|-----------------------|--------------------------------------------------|--------------------------------|--------------------------------|----------------------------------|------------------------|------------------------|-----------------------------------------------------|
| Natural contact      | Off site              | Continuous flow, multiple age groups within room | 950                            | 3                              | N/A                              | 56                     | 5                      | 8/32                                                |
| Aerosolization GDU 1 | On-site quarantine    | All-in, all-out, one age group within room       | 324                            | N/A                            | 4                                | 21                     | 6                      | 7/54                                                |
| Aerosolization GDU 2 | Off-site              | All-in, all-out, multiple age groups within room | 625                            | N/A                            | 4                                | 56                     | 2                      | 20/125                                              |
| Aerosolization GDU 3 | Off-site              | All-in, all-out, multiple age groups within room | 1600                           | N/A                            | 5                                | 35–49                  | 8                      | 5/200                                               |
| Aerosolization GDU 4 | Off-site              | All-in, all-out, multiple age groups within room | 1600                           | N/A                            | 5                                | 35–49                  | 8                      | 5/200                                               |

**Table 2**  
Timeline and sample types collected at each gilt developer unit (GDU).

| Exposure method      | Sample types* collected at days post-exposure |                |                |                |                |    |    |  |
|----------------------|-----------------------------------------------|----------------|----------------|----------------|----------------|----|----|--|
|                      | 0                                             | 14             | 28             | 42             | 56             | 75 | 93 |  |
| Natural contact      | LS, DTS, B, OF                                | LS, DTS, B, OF | LS, DTS, B, OF | LS, DTS, B, OF | LS, DTS, B, OF | B  | B  |  |
| Aerosolization GDU 1 | LS, DTS, B                                    | LS, DTS, B, OF | LS, DTS, B, OF | LS, DTS, B, OF | LS, DTS, OF    |    |    |  |
| Aerosolization GDU 2 | LS, DTS, OF                                   | LS, DTS, OF    |                |                |                |    |    |  |
| Aerosolization GDU 3 | DTS                                           | DTS            | DTS            | DTS, B         | B              |    |    |  |
| Aerosolization GDU 4 | DTS                                           | DTS            | DTS            | B              | B              |    |    |  |

\* LS: laryngeal secretions. DTS: deep tracheal secretions. B: blood. OF: oral fluid.

sampled. Blood samples were collected from all gilts from the jugular vein using vacuum tubes (BD Vacutainer® Blood Collection Tubes, Franklin Lakes, NJ) and sterile needles (Greiner Bio-One™ Multi-Drawing Blood Collection Needles, Munroe, NC). Pen-based oral fluid samples were collected as described by Prickett and Zimmerman (2010) using sterile materials (Web Rigging Supply, Lake Barrington, IL; Corning Science Falcon® 5 mL Polystyrene Round-Bottom Tube, Reynosa, Tamaulipas, Mexico). All samples were refrigerated immediately and submitted to the Health Management Center (HMC; Boehringer Ingelheim Animal Health, Ames, IA) or Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for analysis.

## 2.5. Sample processing

Laryngeal and deep tracheal secretions, and oral fluids were processed for DNA extraction (MagMAX™ Pathogen DNA/RNA Kit and KingFisher™ extraction robot, Life Technologies, Grand Island, NY, USA). Then, a species specific-*M. hyopneumoniae* real-time PCR was performed (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) at the HMC. Samples with Ct values < 38 were considered positive for *M. hyopneumoniae* by real-time PCR.

Blood samples were spun to obtain serum (Sorvall ST40R centrifuge, ThermoFisher Scientific, Waltham, MA), which was tested using an indirect ELISA (Idexx Laboratories, Westbrook, ME) at the ISU VDL. Samples were considered positive when S/P ratio ≥ 0.4. Serum from the natural contact exposure was also tested using a blocking ELISA (Thermo Scientific™ Oxoid™, Grand Island, NY). Samples were

considered positive when OD < 50%.

## 2.6. Data analysis

The proportion of *M. hyopneumoniae* PCR and ELISA positive pigs for each GDU were recorded at each time point, along with the average Ct value and S/P ratio or OD value for positive samples (Ct value < 38; S/P ratio ≥ 0.4; OD value < 50%). The appropriate timing of PCR sample collection for the two methods of gilt exposure was estimated considering different targets of desired proportions of animals testing positive by PCR in deep tracheal secretions.

To develop sample collection schemes to confirm population *M. hyopneumoniae* infection following purposeful exposure, sample sizes were calculated for individual PCR testing. The Freecalc sample size for a finite population and specified cut-point number of positives algorithm (Cameron and Baldock, 1998) function (n.freecalc) from the Surveillance package (Sergeant, 2020) was used to perform the calculations in RStudio (RStudio Team, 2019). A key modification was made to the Freecalc algorithm: since the objective here was the determination of the sample size that would yield a sufficiently high probability of detection of exposed herds in which the proportion of non-infected gilts was above a given threshold (“target herd sensitivity”, SeP, to detect unsuccessfully exposed herds), non-infected animals (either false positive or true negative) were the target animal category of interest. Therefore, sample size required to achieve a given SeP (“n”) was calculated based on “c” (cut-point, number of negative animals to define a herd as “not properly exposed”: if negatives < c herd was successfully exposed, if negatives ≥ c exposure was not successful), Sp (diagnostic specificity), Se (diagnostic sensitivity), pstar (design prevalence of non-infected gilts in the herd that should be detected) and minSpH (minimum desired herd specificity, i.e., minimum probability of correctly identifying a successfully exposed herd).

A perfect specificity was assumed based on previous reports for PCR performance (Strait et al., 2008) and thus no false positive pigs were expected, while the mean (94%, 28 dpi) and 95% lower confidence limit (79%, 28 dpi) of the individual deep tracheal secretions sensitivity for early infection (Sponheim et al., 2020) were alternatively used as unit sensitivity inputs. Since by default n.freecalc assumes that the objective is to detect infected animals/populations, Se and Sp values were entered reversed. The number of individuals to sample were determined for 100, 500, and 1000 population sizes; ≥ 1%, ≥ 5%, ≥ 10%, and ≥ 15% prevalence of non-infected gilt levels; and 85%, 90%, and 95% herd sensitivity and specificity when achievable.

## 3. Results

### 3.1. Detection of *M. hyopneumoniae* genetic material

All diagnostic samples were negative for *M. hyopneumoniae* using

real-time PCR prior to exposure. Detection of *M. hyopneumoniae* by real-time PCR at all samplings for all exposure methods is presented in Table 3. Detection of *M. hyopneumoniae* varied greatly based on sample type. Oral fluids showed the lowest detection in groups of gilts detected

positive by other sample types. Detection of *M. hyopneumoniae* by PCR in deep tracheal secretions was higher than detection in laryngeal secretions. PCR detection in oral fluids was delayed compared to PCR detection in deep tracheal and laryngeal secretions.

**Table 3**  
Detection of *Mycoplasma hyopneumoniae* genetic material and specific antibodies at various sampling time points post-exposure.

| Days post-exposure | Detection target | Sample type               | Diagnostic data                                                   |                     |                    |                     |                    |       |       |          |
|--------------------|------------------|---------------------------|-------------------------------------------------------------------|---------------------|--------------------|---------------------|--------------------|-------|-------|----------|
|                    |                  |                           | # positive/# tested (%); mean Ct value, S/P ratio or %OD ( ± SEM) |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | Exposure method                                                   |                     |                    |                     |                    |       |       |          |
|                    |                  | Natural Contact           | Aerosolization                                                    |                     |                    |                     |                    |       |       |          |
| 0                  | DNA <sup>a</sup> | LS                        | 0/40 (0)                                                          | GDU 1               | 0/42 (0)           | GDU 2               | 0/40 (0)           | GDU 3 | GDU 4 |          |
|                    |                  | DTC                       | 0/40 (0)                                                          |                     | 0/42 (0)           |                     | 0/40 (0)           |       |       | 0/40 (0) |
|                    |                  | OF                        | 0/5 (0)                                                           |                     |                    |                     | 0/2 (0)            |       |       |          |
|                    | Antibody         | B                         | 0/39 (0)                                                          |                     | 0/42 (0)           |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   |                                                                   |                     |                    |                     |                    |       |       |          |
|                    |                  | B (blocking) <sup>c</sup> | 0/39 (0)                                                          |                     |                    |                     |                    |       |       |          |
| 14                 | DNA <sup>a</sup> | LS                        | 5/40 (12.5)                                                       | 1/41 (2.4)          | 38/40 (95)         |                     |                    |       |       |          |
|                    |                  |                           | 34.89 (33.41–36.37)                                               | 33.82               | 32.2 (31.78–32.62) |                     |                    |       |       |          |
|                    |                  | DTC                       | 9/40 (22.5)                                                       | 2/41 (4.9)          | 39/40 (97.5)       | 38/40 (95)          | 34/40 (85)         |       |       |          |
|                    | Antibody         | OF                        | 0/5 (0)                                                           | 0/6 (0)             | 0/2 (0)            |                     |                    |       |       |          |
|                    |                  | B                         | 0/40 (0)                                                          | 0/41 (0)            |                    |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   |                                                                   |                     |                    |                     |                    |       |       |          |
| 28                 | DNA <sup>a</sup> | LS                        | 20/39 (51.3)                                                      | 1/40 (2.5)          |                    |                     |                    |       |       |          |
|                    |                  |                           | 33.83 (33.37–34.29)                                               | 35.55               |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | 24/39 (61.5)                                                      | 1/40 (2.5)          |                    | 33/40 (82.5)        | 40/40 (100)        |       |       |          |
|                    | Antibody         | OF                        | 0/5 (0)                                                           | 0/6 (0)             |                    |                     |                    |       |       |          |
|                    |                  | B                         | 0/39 (0)                                                          | 0/40 (0)            |                    |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   |                                                                   |                     |                    |                     |                    |       |       |          |
| 42                 | DNA <sup>a</sup> | LS                        | 2/39 (5.1)                                                        |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 45.84 (45.37–46.31)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | 32/37 (86.5)                                                      |                     |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        | 32/37 (86.5)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  | B                         | 32.73 (32.19–33.27)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 37/37 (100)                                                       |                     |                    | 40/40 (100)         |                    |       |       |          |
| 56                 | DNA <sup>a</sup> | LS                        | 28.51 (27.87–29.15)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 30.37                                                             |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | 30.37                                                             |                     |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        | (29.5–31.19)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  | B                         | 0/5 (0)                                                           | 0/6 (0)             |                    |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 0/39 (0)                                                          | 0/40 (0)            |                    |                     |                    |       |       |          |
| 75                 | DNA <sup>a</sup> | LS                        | 2/39 (5.1)                                                        |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 45.84 (45.37–46.31)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | 32/37 (86.5)                                                      |                     |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        | 32.73 (32.19–33.27)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  | B                         | 37/37 (100)                                                       |                     |                    | 40/40 (100)         |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 28.51 (27.87–29.15)                                               |                     |                    |                     |                    |       |       |          |
| 93                 | DNA <sup>a</sup> | LS                        | 1/5 (20)                                                          |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 36.57                                                             |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | 1/5 (20)                                                          |                     |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        | 36.57                                                             |                     |                    |                     |                    |       |       |          |
|                    |                  | B                         | 2/37 (5.4)                                                        |                     |                    | 39/40 (97.5)        | 40/40 (100)        |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 0.551 (0.514–0.588)                                               |                     |                    | 1.792 (1.709–1.875) | 1.91 (1.826–1.994) |       |       |          |
| 56                 | DNA <sup>a</sup> | LS                        | 20/37 (54.1)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 33.52                                                             |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | 31.2–35.84)                                                       |                     |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        | 26/37 (70.3)                                                      | 19/39 (48.7)        |                    |                     |                    |       |       |          |
|                    |                  | B                         | 32.52 (31.95–33.09)                                               | 33.93 (33.31–34.55) |                    |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 37/37 (100)                                                       | 30/39 (76.9)        |                    |                     |                    |       |       |          |
| 75                 | DNA <sup>a</sup> | LS                        | 28.28 (27.63–28.93)                                               | 31.81 (31.17–32.45) |                    |                     |                    |       |       |          |
|                    |                  |                           | 31.81 (31.17–32.45)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | 0/5 (0)                                                           | 0/6 (0)             |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        | 17/37 (45.9)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  | B                         | 0.547 (0.518–0.576)                                               |                     |                    | 37/38 (97.4)        | 38/38 (100)        |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 27/37 (73)                                                        |                     |                    | 1.788 (1.713–1.863) | 2.11 (2.038–2.182) |       |       |          |
| 93                 | DNA <sup>a</sup> | LS                        | 26.83 (24.71–28.95)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 26.83 (24.71–28.95)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       |                                                                   |                     |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        |                                                                   |                     |                    |                     |                    |       |       |          |
|                    |                  | B                         | 22/35 (62.9)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 0.815 (0.753–0.877)                                               |                     |                    |                     |                    |       |       |          |
| 93                 | DNA <sup>a</sup> | LS                        | 30/35 (85.7)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 20.53 (18.39–22.68)                                               |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       |                                                                   |                     |                    |                     |                    |       |       |          |
|                    | Antibody         | OF                        |                                                                   |                     |                    |                     |                    |       |       |          |
|                    |                  | B                         | 25/36 (69.4)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  | (indirect) <sup>b</sup>   | 0.461 (0.401–0.521)                                               |                     |                    |                     |                    |       |       |          |
| 93                 | DNA <sup>a</sup> | LS                        | 34/36 (94.4)                                                      |                     |                    |                     |                    |       |       |          |
|                    |                  |                           | 27.79                                                             |                     |                    |                     |                    |       |       |          |
|                    |                  | DTC                       | (26.0–29.58)                                                      |                     |                    |                     |                    |       |       |          |

<sup>a</sup> Real-time PCR. Samples with Ct values < 38 were considered positive.

<sup>b</sup> Indirect ELISA: Samples with S/P ratio ≥ 0.4 were considered positive

<sup>c</sup> Blocking ELISA: Samples with %OD < 50% were considered positive

By two weeks post-exposure,  $\geq 85\%$  of study gilts in Aerosolization GDUs 2, 3, and 4 were PCR positive on the deep tracheal secretions. Aerosolization GDU 1 required a second exposure after the first attempt was determined to be unsuccessful. Four weeks after the second exposure, 77% of study gilts were PCR positive for *M. hyopneumoniae* in the deep tracheal secretions (85% cumulative incidence). Natural contact exposure resulted in 22.5% of study gilts becoming PCR positive by two weeks post-initial exposure, 61.5% at 4 weeks, and 100% at 6 weeks on deep tracheal secretions.

### 3.2. Detection of host immune response

All diagnostic samples were negative for *M. hyopneumoniae* using ELISA prior to exposure. Seroconversion was delayed compared to PCR detection in deep tracheal and laryngeal secretions (Table 3).

### 3.3. Field application

Sample size tables for the number of individuals to sample and cut-point using deep tracheal secretions and *M. hyopneumoniae* PCR during the acute phase of infection for each sampling scheme are presented in Table 4 and Supplementary Table 1. For all scenarios, the number of individuals to sample and cut-point increased as the design (target) prevalence of non-infected gilts decreased, and desired herd sensitivity and specificity increased. At times the desired herd sensitivity and specificity values could not be achieved for given design prevalence (% negatives) at a given population size and a given test sensitivity value.

**Table 4**  
Number of individual pigs to sample and cut-point based on the mean sensitivity of experimentally infected pigs when using deep tracheal secretions early during *Mycoplasma hyopneumoniae* infection<sup>a</sup>.

| Population size | Design Prevalence (% negative) | Herd sensitivity (%) | Herd specificity (%) | Number of individuals to sample | Cut-point |    |
|-----------------|--------------------------------|----------------------|----------------------|---------------------------------|-----------|----|
| 100             | $\leq 1$                       | *                    |                      |                                 |           |    |
|                 | $\leq 5$                       | *                    |                      |                                 |           |    |
|                 | $\leq 10$                      |                      | 85.4                 | 88.6                            | 43        | 5  |
|                 |                                |                      | 89.6                 | 90.3                            | 53        | 6  |
|                 |                                |                      | 95.2                 | 95.3                            | 79        | 9  |
|                 | $\leq 15$                      |                      | 87.0                 | 85.8                            | 22        | 3  |
|                 |                                |                      | 89.1                 | 90.7                            | 29        | 4  |
|                 |                                |                      | 95.4                 | 96.8                            | 51        | 7  |
|                 | 500                            | $\leq 1$             | *                    |                                 |           |    |
|                 |                                | $\leq 5$             |                      |                                 |           |    |
| $\leq 10$       |                                |                      | 86                   | 85.3                            | 142       | 12 |
|                 |                                |                      | 90                   | 90.2                            | 201       | 17 |
|                 |                                |                      | 95                   | 95.4                            | 318       | 27 |
| $\leq 15$       |                                |                      | 85.0                 | 86.9                            | 45        | 5  |
|                 |                                |                      | 90.0                 | 90.5                            | 65        | 7  |
|                 |                                |                      | 95.1                 | 95.2                            | 104       | 11 |
| 1000            |                                | $\leq 1$             | *                    |                                 |           |    |
|                 |                                | $\leq 5$             |                      |                                 |           |    |
|                 | $\leq 10$                      |                      | 85.1                 | 85.4                            | 142       | 12 |
|                 |                                |                      | 88.7                 | 90.2                            | 201       | 17 |
|                 |                                |                      | 94.9                 | 95.0                            | 335       | 28 |
|                 | $\leq 15$                      |                      | 86.0                 | 86.0                            | 46        | 5  |
|                 |                                |                      | 89.6                 | 90.6                            | 65        | 7  |
|                 |                                | 94.5                 | 95.2                 | 104                             | 11        |    |
|                 |                                | 85.0                 | 85.8                 | 22                              | 3         |    |
|                 | 90.7                           | 92.4                 | 38                   | 5                               |           |    |
|                 | 95.4                           | 95.1                 | 56                   | 7                               |           |    |

\* Target herd sensitivity and specificity cannot be achieved for a given design prevalence at the given population size and test sensitivity

<sup>a</sup> Ninety-four percent unit sensitivity at 28 dpi (Sponheim et al., 2020)

## 4. Discussion

This study was designed to determine the appropriate testing approach and timing of sample collection for detection of *M. hyopneumoniae* after two commonly applied methods of gilt exposure. A second objective was to propose sample collection schemes, at various population sizes, population sensitivity and specificities, and prevalence levels, to confirm population *M. hyopneumoniae* infection following purposeful exposure.

Deep tracheal secretions required the lowest number of gilts to be sampled for the earliest detection, compared to all other sample types evaluated. Longitudinal testing suggested that pathogen detection post-exposure varied with the acclimatization method, with at least 85% of study gilts detected by PCR in deep tracheal secretions by two weeks post-exposure in three aerosolization GDUs vs. six weeks in the natural contact GDU. Sampling guidelines are proposed for collection at the expected timing of population exposure based on acclimatization method.

Our results showed that detection of *M. hyopneumoniae* differed based on sample type. Detection by PCR in deep tracheal secretions was higher than detection in laryngeal secretions, which is in agreement with other studies (Sponheim et al., 2020; Betlach et al., 2020; Takeuti et al., 2022) and is consistent with the fact that the trachea and bronchia are considered the multiplication sites of *M. hyopneumoniae* (Blanchard et al., 1992). Oral fluids showed the lowest detection by PCR in groups of gilts positive by other sample types, similar to previous reports (Pieters et al., 2017; Betlach et al., 2020; Clavijo et al., 2021; Poeta Silva et al., 2021), and are a population-based sample type that is not suitable for prevalence-based measurements (Garcia-Morante et al., 2022). Seroconversion and PCR on oral fluids were delayed compared to PCR detection at the individual level, which is also comparable to previous work (Pieters et al., 2017; Betlach et al., 2020; Clavijo et al., 2021; and Poeta Silva et al., 2021). Although the application of PCR and lower airway sample types has added diagnostic cost and labor over serum collection and ELISA testing, this combination is an essential piece of a Day 0 testing scheme to determine the need for re-exposure early in the process.

By two weeks post-exposure, at least 85% of study gilts in three GDUs exposed by aerosolization were detected PCR positive in deep tracheal secretions, which is consistent with Poeta Silva et al. (2021), where 90% of pigs were detected by PCR using deep tracheal secretions at 14 days post-aerosolization. Likewise, Figueras Gourgues et al. (2020) detected 100% of deep tracheal secretion pools of 5 by PCR at 14 days post-aerosolization. McDowell et al. (2023) detected 97% of deep tracheal secretions by PCR at 30 days of herd closure and Nagae et al. (2023) detected 98.1% by PCR at 28 days post-aerosolization and using the same sample type. In contrast, only 4.9% of study gilts in GDU 1 were detected positive by PCR using deep tracheal secretions at 14 days post-exposure. Although the specific reasons for lack of successful exposure in this GDU will never be known, we could hypothesize that differences in the bacterial strain, timeframe between inoculum preparation and administration, and overall conditions of exposure may have played a role in this outcome. Natural contact exposure resulted in 22.5% of study gilts detected by PCR two weeks post-initial exposure, 61.5% at four weeks, and 100% at six weeks on deep tracheal secretions. Natural transmission in this study occurred earlier than estimated by transmission rates determined under experimental conditions (Meyns et al., 2004; Villarreal et al., 2011; Roos et al., 2016; Betlach et al., 2020), what could be due to the method of seeder inoculation, proportion and time that seeders are introduced, contact period, *M. hyopneumoniae* strain, and secondary infections.

Cannon and Roe (1982) developed sample size guidelines for the veterinary community based on perfect tests, which were later modified by Cameron and Baldock (1998), who introduced methods to estimate sample sizes for imperfect tests. Cannon and Roe (1982) guidelines continue to be widely used in the field today. However, they lack

information on the diagnostic accuracy of a given sample type over the course of infection for a specific pathogen, like *M. hyopneumoniae*. The sample collection scheme proposed here is based on the estimated diagnostic sensitivity for deep tracheal secretions early after infection with *M. hyopneumoniae*. For example, following aerosolization exposure of 500 gilts, if a low-risk, low-prevalence level of non-infected gilts approach is targeted using mean diagnostic sensitivity for deep tracheal secretions, sample size estimates for a  $\leq 5\%$  prevalence level of non-infected gilts, 95% herd sensitivity and specificity would be followed since the target herd sensitivity and specificity cannot be achieved for  $\leq 1\%$  design prevalence at the given population size. Three hundred eighteen individuals would be sampled and if  $< 27$  (cut-point) samples were PCR negative, the selected criteria would be achieved. If  $\geq 27$  samples were PCR negative, the chosen criteria would not be achieved, and contingency plans should be discussed, such as delaying Day 0, resampling, or repeating the exposure method. If a higher prevalence of non-infected gilts is assumed, sample size estimates for  $\leq 15\%$  design prevalence of non-infected gilts could be followed. Fifty-five individuals would be sampled and if  $< 7$  (cut-point) samples were PCR negative, the selected criteria would be achieved. If  $\geq 7$  samples were PCR negative, contingency plans would be followed. Samples could be collected as early as 14 days post-aerosolization exposure.

Sample size selection for validation of population exposure relies on several assumptions, including a reasonable target for within-herd prevalence. When using imperfect diagnostic methods, confirmation of complete population exposure (i.e., 0% prevalence of non-infected gilts) may not be achievable even if all individuals are sampled, and the limitations of the sampling strategy should be understood. If design prevalence of  $\leq 15\%$  non-infected gilts is selected, consideration should be made to add time between sample collection and Day 0 for *M. hyopneumoniae* transmission to the remaining non-infected gilts. The deep tracheal secretion diagnostic sensitivities used in this study are the highest reported for any in vivo sample type early in infection and are representative of the performance of the test when applied in experimentally infected pigs at 28 days post-inoculation (Sponheim et al., 2020). It would be ideal to consider diagnostic sensitivity estimates from an even earlier stage of *M. hyopneumoniae* infection if diagnostic sampling occurs prior to 28 days post-aerosolization. It is important to note that the sample guidelines provided here only consider three GDU population sizes in North America, and even if these are common sizes in this region, all GDU scenarios are not covered. Further studies underway are directed at validating the sample collection schemes in the field, adapting a transmission model to estimate time to GDU population exposure based on diagnostic outcome, and developing ongoing surveillance programs for *M. hyopneumoniae* negative herds.

In conclusion, these results suggest that *M. hyopneumoniae* Day 0 sampling by deep tracheal secretions and PCR may occur as early as two weeks following aerosolization exposure and at least six weeks for natural contact. Furthermore, the proposed sample collection scheme can be applied in commercial settings for verification of *M. hyopneumoniae* infection of gilts following exposure to determine “Day 0” of herd closure or to initiate a contingency plan. This information will aid veterinarians and producers to provide certainty and make better-informed decisions when setting a timeline for herd closure.

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## Declaration of Competing Interest

Boehringer Ingelheim Animal Health USA, Inc. provided funding for the study and employs Drs. Sponheim and Fano. However, the evaluation of commercial products was not part of this investigation.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.prevetmed.2023.106057](https://doi.org/10.1016/j.prevetmed.2023.106057).

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