Comparison of Mycoplasma hyopneumoniae response to infection by route of exposure

Ana Paula S. Poeta Silva  
*Iowa State University*, apsilva@iastate.edu

Thaire P. Marostica  
*Universidade Federal de Minas Gerais*

Aric McDaniel  
*Iowa State University*

Bailey L. Arruda  
*Iowa State University*

Carmen Alonso  
*Independent swine health consultant*

*See next page for additional authors*

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Abstract
Mycoplasma hyopneumoniae (MHP) is a concern both for pig well-being and producer economic viability. In the absence of fully protective health interventions, producers rely on controlled exposure to induce an immune response in pigs and minimize the clinical outcomes of MHP infection in pig populations. This study compared the effect of route of exposure on MHP infection, antibody response, clinical signs, and pathology. Six-week-old MHP-negative pigs (n = 78) were allocated to negative control (n = 6) or one of three MHP exposure routes: intratracheal (n = 24, feeding catheter), intranasal (n = 24, atomization device), and aerosol (n = 24, fogger). Body weight, cough indices, and samples (serum, oral fluid, tracheal) were collected weekly through 49 days post-exposure (DPE). Intratrachal exposure produced the highest proportion (24/24) of MHP DNA-positive pigs on DPE 7, as well as earlier and higher serum antibody response. Intranasal and aerosol exposures resulted in infection with MHP DNA detected in tracheal samples from 18/24 and 21/24 pigs on DPE 7, respectively. Aerosol exposure had the least impact on weight gain (0.64 kg/day). No difference was observed among treatment groups in coughing and lung lesions at necropsy. While intratracheal inoculation or the use of seeder animals are frequently used in swine production settings, intranasal or aerosol exposure are viable alternatives to achieve infection. Regardless of the route, steps should be taken to verify the purity of the inoculum and, in the case of aerosol exposure, avert the unintended exposure of personnel and animals to other pathogens.

Keywords
intratracheal, intranasal, aerosol, exposure, flow rate, oral fluids

Disciplines
Large or Food Animal and Equine Medicine | Veterinary Infectious Diseases | Veterinary Microbiology and Immunobiology

Comments

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Comparison of *Mycoplasma hyopneumoniae* response to infection by route of exposure

Ana Paula S Poeta Silva¹, Thaire P Marostica², Aric McDaniel¹, Bailey L Arruda¹, Carmen Alonso³, Rachel Derscheid¹, Paul Yeske⁴, Daniel C L Linhares¹, Luis Giménez-Lirola¹, Locke Karriker¹⁵, Eduardo Fano⁶, Jeffrey J Zimmerman¹, Maria J Clavijo¹⁷

¹ Veterinary Diagnostic and Population Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, U.S.A.
² Departamento de Clínica e Cirurgia Veterinárias, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brasil
³ Independent swine health consultant, Barcelona 08195, Spain
⁴ Swine Vet Center, P.A., St. Peter, MN, U.S.A.
⁵ Swine Medicine Education Center, College of Veterinary Medicine, Iowa State University, Ames, IA, U.S.A.
⁶ Boehringer Ingelheim Animal Health US Inc., Atlanta, GA, U.S.A
⁷ Pig Improvement Company, PIC®, Hendersonville, TN, U.S.A.

Corresponding author.

E-mail address:mclavijo@iastate.edu (M.J. Clavijo). Present address: Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, U.S.A / Pig Improvement Company, Hendersonville, TN, U.S.A.
Highlights

- All routes of exposure resulted in similar MHP infection by 21 days post exposure.
- Intratracheal exposure resulted in earlier and higher antibody response
- Aerosol exposure had the least impact on average daily weight gain.
- Intranasal or aerosol routes are viable alternatives to achieve infection.
- All routes of exposure require attention to the purity and quality of the inoculum

Abstract

Mycoplasma hyopneumoniae (MHP) is a concern both for pig well-being and producer economic viability. In the absence of fully protective health interventions, producers rely on controlled exposure to induce an immune response in pigs and minimize the clinical outcomes of MHP infection in pig populations. This study compared the effect of route of exposure on MHP infection, antibody response, clinical signs, and pathology. Six-week-old MHP-negative pigs (n = 78) were allocated to negative control (n = 6) or one of three MHP exposure routes: intratracheal (n = 24, feeding catheter), intranasal (n = 24, atomization device), and aerosol (n = 24, fogger). Body weight, cough indices, and samples (serum, oral fluid, tracheal) were collected weekly through 49 days post-exposure (DPE). Intratrachal exposure produced the highest proportion (24/24) of MHP DNA-positive pigs on DPE 7, as well as earlier and higher serum antibody response. Intranasal and aerosol exposures resulted in infection with MHP DNA detected in tracheal samples from 18/24 and 21/24 pigs on DPE 7, respectively. Aerosol exposure had the least impact on weight gain (0.64 kg/day). No difference was observed among treatment groups in coughing and lung lesions at necropsy. While intratracheal inoculation or the use of seeder animals are frequently used in swine production settings, intranasal or aerosol exposure are viable alternatives to achieve infection. Regardless of the route, steps should be
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**Keywords:** intratracheal, intranasal, aerosol, exposure, flow rate, oral fluids

**Introduction**

*Mycoplasma hyopneumoniae* (*MHP*) is the primary agent of porcine enzootic pneumonia (Mare and Switzer, 1965). *MHP*-infected pigs exhibit chronic respiratory disease (Thacker et al., 1999), lower average daily weight gain, and poor feed conversion (Maes et al., 2018), significantly affecting pig well-being and diminishing the productivity of swine production systems. Once introduced into the respiratory system, *MHP* adheres to the cilia on the epithelium of trachea, bronchi, and bronchioles (Seymour et al., 2012) and then compromises the normal function of the muco-ciliary apparatus. The clinical effects of *MHP* are exacerbated in the presence of other pathogens, i.e., porcine reproductive and respiratory syndrome virus (PRRSV) (Thacker et al., 1999) or porcine circovirus 2 (Opriessing et al., 2004). Gillespie et al. (2013) estimated the cost of an *MHP* outbreak in a *MHP*-naïve population at $8 per pig and Dykhuis-Haden et al. (2008) calculated a cost of ~$10 per pig when *MHP* infections occurred in combination with PRRSV or influenza A virus.

Two key characteristics of *MHP* epidemiology are the slow rate of transmission and long term persistence within individual animals. In commercial swine herds, the ecology of *MHP* is maintained through a cycle in which piglets are farrowed free of *MHP*, but some piglets acquire
the infection from sows or infected suckling pigs during lactation. Thereafter, the infection moves from infectious-to-susceptibles in the growing pig population (Fano et al., 2007; Sibila et al., 2008). Meyns et al. (2004) and Roos et al. (2016) estimated a MHP reproduction ratio of 1.16, and a transmission rate of 1.28 pigs per week over a 4-week period of production, but due to its long incubation period, practitioners are more likely to observe clinical signs of MHP in grow-finish pigs (Nathues et al., 2013). This cycle is facilitated by the fact that MHP is a chronic and prolonged infection in which carrier pigs become a source of MHP for susceptible animals (Pieters et al., 2009). Pieters et al. (2009) detected MHP DNA in tracheal samples for 214 days post inoculation under experimental conditions. Consequently, identification of MHP-infected animals is a crucial step in control and prevention.

Data have shown that MHP positive gilts and young sows are the main source of the pathogen to piglets (Pieters et al., 2014). Thus, the goal in the MHP control programs is to induce MHP immunity and provide time for the immune system to clear the infection from the animal prior to her first farrowing so that her piglets are not exposed/infected during lactation (Pieters and Fano 2016). Strategic vaccination is commonly used to achieve this purpose (Garza-Moreno et al., 2019). Although vaccines might significantly improve growth performance and reduce clinical signs and lung lesion at slaughter caused by MHP infection (Maes et al., 2018), shedding from infected animals and colonization of the respiratory tract of susceptible animals still occurs (Sibila et al., 2008).

An alternative approach to achieving robust immunity relies on infecting MHP-susceptible animals (Pieters and Fano, 2016; Yeske, 2017). This may be done by commingling with MHP-
positive animals, i.e., promoting pig-to-pig natural transmission (seeder pig model), but the slow transmission of MHP, flow constraints, and the high number of "MHP seeders" (Roos et al., 2016) required for exposure of a population renders this approach unreliable and impractical due to the time required for complete exposure under field conditions (Yeske, 2017). Given that gilt replacement occurs at ~45% per year (Kraeling and Webel, 2015), controlled exposure merits consideration for ensuring infection of an entire group of pigs at the same point in time. As described elsewhere (Sörensen et al. 1997; Garcia-Morante et al., 2016), this objective can be achieved by intratracheal, intranasal, and aerosol inoculation using cultures of MHP. However, producing a sufficient concentration of number of MHP pure colonies MHP can be an obstacle, given the fastidious nature of MHP for isolation and propagation using current laboratory techniques (Thacker, 2004; Garcia-Morante et al., 2018). Furthermore, various MHP isolates circulate in pig populations (Nathues et al., 2011), hence, identifying a "standard MHP inoculum" to be used across swine farms is problematic.

Recent reports have described alternative methods for achieving more reliable and uniform MHP infections in the field, including the use of farm-specific MHP lung homogenates administered intratracheally or via aerosol (Robbins et al., 2019; Yeske, 2017). However, there are few comparisons of inoculation procedures in terms of clinical impact, antibody response, and consequences on productivity. Therefore, the objective of this study was to compare intratracheal, intranasal, and aerosol MHP exposure protocols in terms of MHP shedding, antibody response, pathology, clinical signs, and growth rate under controlled conditions.
Materials and methods

2.1 Experimental design

Six-week-old gilts originating from a known PRRSV and *MHP* negative herd were assigned to one of 4 treatments, each in a separate room: 1) negative controls (n = 6 pigs); 2) aerosol exposure to *MHP* (n = 24); 3) intranasal exposure to *MHP* (n = 24); and 4) intratracheal exposure to *MHP* (n = 24) and monitored for 49 days. *MHP* infection status was determined using blood samples for antibody testing and deep tracheal samples for DNA by real-time polymerase chain reaction (PCR) testing on a weekly basis. In addition, oral fluid and environmental air samples for DNA testing were collected at 3- to 4-day intervals. Pigs were humanely euthanized on 49 day post exposure (DPE) and evaluated for gross and microscopic pulmonary lesions. Average daily weight gain, antibody responses (ELISA sample-to-positive ratio, S/P), and total *MHP* shedding (PCR Ct values) were analyzed using linear mixed regression and area under curve (AUC). Differences among groups were evaluated by ANOVA. All procedures were conducted with the approval of the Iowa State University Office for Responsible Research and Institutional Animal Care and Use Committee (# 18-194).

2.2 Animal housing

Animals (n = 78) were housed in a BSL-2 livestock infectious disease isolation facility equipped with a single-pass non-recirculating ventilation system for the prevention of aerosol transmission. Upon arrival, pigs were blocked by weight and randomly allocated to 4 groups in 4 separate rooms (each 5.48 m × 10.97 m × 3 m): 1) negative controls (6 pigs), 2) aerosol exposure to *MHP* (n = 24), 3) intranasal exposure to *MHP* (n = 24), and 4) intratracheal exposure to *MHP* (n = 24). Sample size (24 pigs per inoculum) was based on the expectation of detecting
a significant difference between proportions (95% confidence level, 80% power, one-tailed test) under the assumption that 99% of pigs would become infected by intratracheal exposure and 70% of pigs would become infected via aerosol or intranasal exposures (Epitools epidemiological calculators at http://epitools.ausvet.com.au). Within rooms, pigs were housed in 2 pens (n = \leq 12 animals per pen).

2.3 *Mycoplasma hyopneumoniae* inoculation

Prior to *MHP* inoculation, pigs were confirmed to be free of *MHP* infection by testing serum and deep tracheal samples for *MHP* antibodies and nucleic acids, respectively. For intratracheal and intranasal inoculation, *M. hyopneumoniae* (strain 232) inoculum consisted of lung tissue homogenate (Lot 44, Veterinary Diagnostic Laboratory, Iowa State University, ISU-VDL) from *MHP*-inoculated cesarean derived, colostrum deprived pigs diluted in 1:100 Friis broth medium (ISU-VDL Doc 9.6726) to a concentration estimated at $1.0 \times 10^5$ CCU per mL by titration. For the aerosol inoculation, *M. hyopneumoniae* (strain 232) inoculum consisted of lung tissue homogenate but initially concentrated at $1.0 \times 10^7$ CCU per mL by titration and then suspendend at 1:100 in Friis broth medium (Friis Media Broth, Teknova Inc., Hollister, CA USA). The final concentration of 1 L *MHP* lung homogenate mixture was estimated at $1.0 \times 10^5$ CCU per mL by titration.

2.3.1 Intratracheal inoculation

After restraining pigs with a nasal snare, the intratracheal inoculation was performed by using an oral speculum to open the mouth of a restrained animal, depressing the tongue with a laryngoscope, introducing a feeding tube/urethral catheter (Integral Funnel, Two Eyes, Rounded
Closed Tip, 4.7 mm x 41 cm, COVIDEN™ Kendall™, Coviden Ilc, Mansfield, MA USA) into the larynx, and then administering the *MHP* inoculum (10 mL).

2.3.2 Intranasal inoculation

Pigs were restrained manually and intranasal inoculation was performed by administering the *MHP* inoculum (5 mL per nostril) using a laryngo-tracheal mucosal atomization device (MADgic®, model MAD720, 4.8 mm x 12.4 cm, Teleflex, Morrisville, NC USA) attached to a syringe. According to the manufacturer, the device generates droplets 30–100 µm in diameter (http://www.lmaco.com/sites/default/files/940696-000001_MADgic_Data-Sheet_1607_PDFonly.pdf). Administration of the intranasal inoculum was timed to coincide with pig inhalation to ensure deposition of *MHP* in the tissues required for colonization.

2.3.3 Aerosol inoculation

Aerosols of *MHP* were generated using a fogger (Dyna-Fog® Hurricane "Cold Fog" ULV/Mister model 2792, Westfield, IN USA) operated according to the manufacturer's instructions. Based on the manufacturer's instruction manual, the device generates aerosols 7 - 30 µm in diameter at three flow rates: low (189 mL per min), medium (236 mL per min), and high (266 mL per min) when aerosoziling water. The number and size of airborne particles generated by the fogger per cubic meter (air particles per m³) were estimated using an optical particle counter (AeroTrak™ Handheld Particle Sizer Model 9306-04, TSI Incorporated, Shoreview, MN USA). The optical particle counter device classified particles into the following ranges: < 0.3, 0.3 to < 0.5, 0.5 to <1.0, 1.0 to <3.0, 3.0 to < 5.0, and 5.0 to < 10.0 µm in diameter.

To evaluate the presence of infectious *MHP* and total *MHP* DNA in the aerosols generated, air
samples were collected using a 6-stage Andersen Cascade Impactor (Series 10-800, Thermo Fisher Scientific Inc, Franklin, MA USA) and a SKC BioSampler® impinger (SKC Inc., Eighty Four, PA USA) operating simultaneously for the entire fogging event (Section 2.5.3).

To determine the optimum flow rate used by the fogger during the animal experiment, a pilot study was carried out in one room (5.48 × 5.48 × 3 = 90.1 m³) in a BSL-2 facility over the course of two days. Each of the 3 flow rates were each tested in 3 replicates (one replicate of a given flow rate represented a “fogging event”) using MHP lung homogenate mixture (1 L), i.e., MHP lung homogenate (10 mL) suspended in commercial Friis broth medium (990 mL, Friis Media Broth, Teknova Inc., Hollister, CA USA) at a MHP concentration estimated at 1.0 × 10⁵ CCU per mL. The flow rate of the device was calculated as the volume of lung homogenate aerosolized over the time of aerosolization for each of the meter value settings (low, medium, high). Airborne particle sizes were measured continuously by the optical particle counter for 15 min prior to flow rate testing (baseline), 15 min during the fogging event, and then 15 min after aerosolization for each flow rate replicate. Temperature and relative humidity were measured before and after each replicate. Differences in airborne particles per m³ fogged across flow rates were assessed by linear regression.

For the animal experiment, the fogger, filled with the lung homogenate mixture (1.980 L of Friis medium and 20 mL of MHP lung homogenate) was placed 1.5 m above the floor, directed toward the center of the room (180.35 m³), and was operated for a period of 18 min using the medium flow rate. Ventilation inlets were sealed to minimize loss of aerosol and the animals were encouraged to move within the pens by personnel standing next to the pens while wearing
adequate protective equipment (glasses, mask, coveralls, and gloves), to increase respiratory rate and ensure inhalation of the *MHP* aerosol. Airborne particle sizes were measured by the optical particle counter for 15 min prior to flow rate testing (baseline) and then 15 min during the fogging event of the *MHP* lung homogenate. Temperature and relative humidity were measured before and after fogging.

2.2.1 Clinical observations

Body weight (kg) was recorded on -1, 28, and 49 DPE. Animals were monitored for coughing twice per week beginning on DPE 3. Prior to cough evaluation, the animals were stimulated to stand and walk for ~1 min. Thereafter, coughs per room were counted for 3 min and scored using a "cough index calculator" ([https://www.preventionworks.info/en/support-tools/Cough-Index-App](https://www.preventionworks.info/en/support-tools/Cough-Index-App)). Cough indices were based on the total counts of coughs in 3 min over the number of observed pigs, with indices >2.5 suggestive of respiratory compromise (Nathues et al., 2012). The negative control group (n = 6 pigs) was evaluated for coughing using the same method, but the cough index calculator was not used because the software required a minimum of 20 pigs for correct function.

2.5 Sample collection and processing

2.5.1 Tracheal samples: Deep tracheal and blood samples were collected weekly (-1, 7, 14, 21, 28, 35, and 42 DPEs) from all animals. Deep tracheal samples were collected using a sheathed, single-use porcine cervical artificial insemination catheter (Nasco, Fort Atkinson, WI USA). Immediately after sampling, the tip containing the collected material was severed from the catheter and placed in a tube containing 1 mL sterile PBS. In the laboratory, samples were
transferred to 2 mL cryogenic tubes (Cryo.s™, Greiner Bio-One™) for storage at -80 °C. After collecting tracheal samples, blood samples were collected using a single-use system (BD Vacutainer™, Fisher Scientific, Hampton, NH USA). In the laboratory, samples were centrifuged (1,500 × g for 10 min at 4°C) and sera aliquoted into 2 mL cryogenic tubes (Cryo.s™, Greiner Bio-One™, Monroe, NC USA) for storage (-20°C).

2.5.2 Oral fluids: Pen-based oral fluid samples were collected before animal exposure on DPE 0 and then twice weekly (DPEs 4, 8, 11, 15, 18, 22, 25, 29, 32, 36, 39, 43, 46) using 3-strand (1.6 cm), 100% cotton rope (Web Rigging Supply, Lake Barrington, IL USA) suspended from a bracket fixed to the side of the enclosure. Samples were recovered from the rope after 15 min exposure to the animals. In the laboratory, oral fluid samples were transferred to 4 mL cryogenic tubes (Cryo.s™, Greiner Bio-One™), and stored at -80°C.

2.5.3 Aerosol samples: 9 replicas of flow study (3 replicas per flow rate, low, medium, and high), and one replicas at the first 15 min of the aerosol exposure on DPE 0 were collected using a 6-stage Andersen Cascade Impactor (Series 10-800, Thermo Fischer Scientific Inc) and a SKC BioSampler® impinger (SKC Inc.) operating simultaneously for 15 min. Vacuum pressure for the Andersen Cascade Impactor was maintained using a jar-less sampling pump (Series 9801-88 T-100, Allegro Industries Inc, Piedmont, SC USA) and an oil-less sampling pump for the BioSampler® (Cat No. 228-9605m, VAC-U-GO Air Sampler, SKC Inc.). The Andersen Cascade Impactor (28.3 L per min) differentially separated particles into 6 sizes (0.8, 1.6, 2.7, 4.0, 5.8, and >7.0 μm in diameter). Collected air particles of specific sizes were then impacted onto one of 6 agar plates (Friis medium agar-plate, ISU-VDL Doc 9.6726), which corresponded to the stage of specific air particle size. The impinger (12.5 L per min) was loaded with 20 mL of Friis medium broth.
ISU-VDL Doc 9.6726) and airborne particles were recovered through impingement. Between use, air samplers were disinfected with alkyl dimethyl benzyl ammonium chloride soap (Lysol®, Reckitt Benckiser, Slough UK), rinsed, and dried.

To prepare samples for PCR and culture, the entire surface of each Andersen Cascade Impactor plate (6 agar-plates per sampling event × 3 replicas × 3 flow rates = 54 samples tested by *MHP* culture and PCR) was swabbed with a cotton-tipped (Fisherbrand™ Applicator, Thermo Fischer Scientific Inc) pre-moistened in a tube containing PBS (0.9 mL). The swab was then returned to the same PBS tube and the solution tested by PCR (200 µL) and cultured for *MHP*. Friis medium recovered from the impinger (1 sample per sampling event × 3 replicas × 3 flow rates = 9 samples tested by *MHP* culture and PCR) was likewise tested by PCR (200 µL) and cultured for *MHP*.

2.5.4. Post-mortem examination

At necropsy, lungs were evaluated and scored for gross lesions using a procedure as described elsewhere (Halbur et al., 1995). In brief, each lung lobe was evaluated for visible areas of consolidation, i.e., well-demarcated dark red-to-purple tissues, and given a total lesion score by summing the contribution of each lobe to the entire lung volume. The right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe were each considered to constitute 10% of the total lung volume, the accessory lobe 5%, and the right and left caudal lobes 27.5% each. Lung tissue samples containing both normal and affected areas (3 × 3 cm) were collected for *MHP* DNA testing and histopathology. Lung tissue samples for histopathological examination were placed in 10% neutral buffered formalin. Samples for
MHP PCR were stored at -80°C until processed. For evaluation of histopathology and immunohistochemical (IHC) detection of MHP on lung tissue, formalin-fixed lung tissues were routinely processed and embedded in paraffin wax. Paraffin slides were sectioned (4 µm) and stained with hematoxylin and eosin (H&E) using routine procedures.

2.6 Sample testing

2.6.1 Mycoplasma hyopneumoniae PCR

For the MHP PCR, tissues were thawed at room temperature, minced with sterile scissors, and then placed in a 50 mL conical tube with 30 mL of Earle’s Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO USA) at a concentration of 10% (weight by volume). Thereafter, the sample was homogenized (2 min at 1000 rpm; Geno/Grinder®, SPEX® SamplePrep, Metuchen, NJ USA), centrifuged (10 min at 4200 × g), and held at 4°C until tested by PCR. MHP DNA in air, deep trachea, oral fluid, and lung tissue samples was extracted using MagMAX™-96 Pathogen RNA/DNA kit (Applied Biosystems™, Carlsbad, CA USA) on the Kingfisher™ Flex System (Thermo Fisher Scientific, Waltham, MA USA). Thereafter, DNA was amplified using TaqMan® Fast Virus 1-Step Master Mix (Life Technologies, Carlsbad, CA USA) with primers and probes described for MHP183 (Strait et al., 2008) on Applied Biosystems® 7500 Real-Time PCR (Thermo Fisher Scientific). A test result was considered valid when the internal positive cycle threshold (Ct) value was ≤ 36. A sample was considered MHP-positive when Ct values were < 37.

2.6.2 Mycoplasma hyopneumoniae culture from air samples
**MHP** titration of air samples (flow trial and aerosol inoculation) was done using a color-changing units (CCU per mL) method. Briefly, air samples collected using the Andersen Cascade Impactor and impinger were serially 10-fold diluted (1 × 10^4 to 1 × 10^5) using Friis medium broth containing a pH sensitive color indicator as the diluent. Tubes were sealed and incubated (37°C, 5% CO₂) until growth was observed, i.e. a color change from pink-to-red to orange-to-yellow by pH acidification due to **MHP** metabolic activity. The titer was considered that highest dilution at which color change was observed.

### 2.6.3 Mycoplasma hyopneumoniae antibody

Serum samples were tested using a commercial serum antibody ELISA (M hyo Ab Test, IDEXX Laboratories Inc., Westbrook, ME USA) using the procedure provided by the manufacturer. Results were given in terms of ELISA S/P ratio and interpreted as S/P < 0.3 = negative and S/P ≥ 0.3 = positive. Suspect samples were considered positive. This differs from the manufacturer's recommendations, but is justified in that “suspect” classification is not a conclusive result – pig is either serum antibody **MHP** positive or negative (Poeta Silva et al., 2020). Therefore, in this study, results with “suspect” classification were assumed as serum antibody **MHP** positive.

### 2.6.4 Mycoplasma hyopneumoniae immunochemistry

For immunohistochemical (IHC) detection of **MHP** on paraffin-embedded lung tissues, the 4 um paraffin sections were placed on negatively charged glass slides and dried for 20 min at 60°C. Thereafter, samples were dewaxed on the automated Leica BOND RX staining platform (Leica Biosystems, Microsystems Inc., Buffalo Grove, IL USA) following manufacturer’s instructions. Upon dewaxing, antigens were retrieved using the BOND Epitope Retrieval Solution 2 (Leica
Biosystems) incubated for 20 min at 100°C, followed by application for 15 minutes of *MHP* primary antibody D79a (ISU-VDL) diluted at 1:100 in Primary Antibody Diluent (Leica Biosystems). After rinse, secondary HRP anti-mouse IgG polymer was added for 25 min. Endogenous peroxide blocking was achieved by 7 min incubation with 3% hydrogen peroxide (Fisher Fisher Scientific), followed by chromogen detection using DAB Refine Kit (Leica Biosystems) for 10 min, and counterstaining with hematoxylin for 5 min. Slides were then removed from the automated Leica BOND RX staining platform, and placed on the Tissue-Tek Prisma® Automated Slide Stainer (©Sakura Finetek USA, Inc., Torrance, CA USA), where slides were linked to the Tissue-Tek® Glas™ g2 Automated Glass Coverslipper for automated dehydration and coverslipping.

2.6.5 *Mycoplasma hyopneumoniae* histopathologic evaluation

Microscopic lung lesions were scored based on the degree of hyperplasia of bronchus-associated lymphoid tissue (BALT) surrounding conducting airways and the presence of peribronchiolar infiltrate of lymphocytes. Lung lesion scores ranged from 0 to 4, where 0 = No lesions, 1 = Mild BALT OR peribronchiolar infiltrate, 2 = Mild peribronchiolar infiltrate, minimal to mild BALT, 3 = Moderate BALT and peribronchiolar infiltrate, 4 = Marked BALT hyperplasia with distortion of airway and peribronchiolar infiltrate. Scores of 3 or 4 are consistent with a diagnosis of mycoplasmosis. Scores for IHC ranged from 0 to 3, 0 = no labeling, 1 = scant labeling of one or more airways, 2 = moderate labeling of one or more airways, and 3 = intense labeling on the surface of one or more airways.
2.7 Statistical analysis

Analyses and graphs were performed in R (R program version 3.6.0, R core team 2019), and SAS (SAS v.9.4, SAS Institute, Cary, NC USA). An alpha level ≤ 0.05 (p ≤ 0.05) was considered significant. Residuals and variances of errors from linear mixed models and ANOVA were evaluated for normality and homoscedasticity assumptions using Q-Q plots, Shapiro-Wilk test (p > 0.05), and Residuals versus Fittes plots. The flow study provided estimates on the number of airborne particles per m$^3$ for 3 flow rates (low, medium and high) at 30-second intervals for 30 time points. These data were analyzed using a linear mixed model with air particles per m$^3$ (geometric mean) as the dependent variable, flow rate as a fixed effect, and time as the random effect.

Data on individual pigs (n = 78) included body weight (3 time points), $MHP$ PCR Ct values on deep trachea samples (6 time points) and lung tissue samples (post-mortem), $MHP$ serum ELISA S/P ratios (7 time points each), and evaluations of lung pathology (gross and microscopic). At the group level (n = 4), data included cough indices and oral fluid $MHP$ PCR Ct values (24 time points each).

The body weight response was analyzed for each pig using a linear mixed model with body weight as the dependent variable, DPE as the fixed effect, and pig as a repeated measure. Random slopes and intercepts were then compared among the 4 groups using ANOVA, followed by Tukey-Kramer pair-wise comparisons ($lme4$ and $emmeans$ R packages).
Total *MHP* shedding by exposure route was compared using an area under curve (AUC) analysis (*caTools* R package) with DPE as the x-axis (-1, 7, 14, 21, 28, 35, and 42 DPEs) and adjusted *MHP* PCR Ct values of deep tracheal samples as the y-axis. "Adjusted Ct" was defined as (37 - sample Ct). The AUC was calculated for each animal as the integral of the displayed shape based on the trapezoidal rule, i.e., PCR positive results (Ct<37) at 6 time points. Thereafter, AUCs were compared among the 3 groups by ANOVA followed by a Tukey-Kramer post-hoc test (*emmeans* R package).

The variation of ELISA S/P ratios for a given DPE were compared among routes of exposure using linear mixed regression (PROC GLIMMIX, SAS). ELISA S/P ratios were adjusted (negative signal was ignored) and included as the dependent variable, *MHP* exposure route, DPEs (-1, 7, 14, 21, 28, 35, 42, and 49 DPEs as categorical variable), and interaction (routes of exposure × DPEs) as fixed effects, with pigs included as the random effect. The linear mixed regression was followed by Tukey-Kramer test to pairwise comparisons of ELISA S/P ratios.

PCR Cts from lung tissues were compared among the 3 *MHP*-exposed groups by ANOVA followed by a Tukey-Kramer post-hoc test (*emmeans* R package). Evaluation of lung lesion scores and cough indices revealed a lack of linearity of model residuals (Shapiro-Wilk W test, p < 0.05). Therefore, the effect of *MHP* exposure routes on gross and microscopic lung lesions (HE and IHC) and cough indices were assessed by using a non-parametric statistical method, Kruskall-Wallis test, followed by Dunn Test for pairwise comparisons (*FSA* R package).
To account for the lack of normal distribution of PCR Ct values and fluctuations in oral fluid PCR testing results over time and between pens within treatments, the effect of *MHP* exposure was analyzed in terms of the positivity rate by mixed logistic regression (*lme4* and *emmeans* R packages). Binary PCR results (positive or negative) were used as the dependent variable, *MHP* exposure route as the fixed effect, and group identification and pen were considered random effects.

**Results**

3.1. Flow study

A summary of results by flow rate is shown in Table 1. Prior to and after fogging, room temperature and relative humidity were 22°C / 46% and 21°C / 75%, respectively. The mean time to deliver 1 L of suspended lung homogenate was estimated at 4.5 min (high flow), 9 min (medium flow), and 19 min (low flow). A total of 40 of 54 samples collected with the 6-stage Andersen Cascade Impactor were both *MHP* culture and DNA positive, i.e., all three replicas of air particle of size 1.6, 2.7, 4.0, 5.8, and >7.0 µm in diameter from the three tested flow rates resulted in positive results, with the exception of all air particles samples of 0.8 µm in size (n = 9 samples) and one entire replica from the low flow rate (n = 5 samples comprising 1.6, 2.7, 4.0, 5.8, and >7.0 µm). A total of 8 of 9 samples collected with the impinger were both *MHP* culture and DNA positive, with exception of one replica from the low flow rate.

Based on optical particle counter measurements, (Table 1, Figure 1), the number of airborne particles per m³ of air was associated with flow rate (p ≤ 0.05, Tukey-Kramer) and, with the exception of particles 0.8 µm in size, viable *MHP* (culture positive) and *MHP* DNA (PCR
positive) was associated with all particle sizes (1.6, 2.7, 4.0, 5.8, and >7.0 µm). Furthermore, visualization of the geometric mean of air particles per m³ of air over time (Figure 1) showed that the number of suspended particles was relatively consistent over time, particular in aerosols generated with low and medium flow rates. Based on these results, the medium flow was selected for the animal experiment on the basis of the time required to deliver the inoculum, number of air particles per m³ of air, patterns of aerosols over time, and detection of MHP by culture and PCR.

3.2 Experimental inoculations

Negative control animals (n = 6) remained free of MHP infection as shown by negative MHP PCR testing of tracheal, oral fluid, and lung tissue samples and the absence of detectable antibody in serum samples. Measurements taken in association with aerosol exposure showed that room temperature and relative humidity were 25°C and 49% prior to fogging and 25°C and 94% after fogging. The concentration of MHP airborne particles per m³ of air was estimated at $4.43 \times 10^3$ (geometric mean). MHP culture was not achieved due to contamination from air samples collected with the impinger and Andersen Cascade Impactor. All animals in intratracheal (n = 24), intranasal (n= 24), and aerosol (n = 24) exposure groups became infected, as evidenced by the detection of MHP DNA and rising MHP antibody over time post exposure (Figure 2).

3.2.1 Clinical observations

One pig in the intratracheal exposure group died after blood sampling on DPE 36 and one pig in the same group was found dead on DPE 48 and was diagnosed with pericarditis at necropsy.
(Streptococcus suis isolated from heart and fibrin specimens with the isolate then tested PCR positive for Streptococcus suis serotype 31).

Mean body weight on DPE -1 (26.8 kg, SD = 4.4 kg, n = 78) did not differ among exposure groups (p > 0.05, Tukey-Kramer). Over the course of the study all MHP exposure groups had numerically lower rates of weight gain, i.e., intratracheal (0.58 kg per day, 95% CI 0.54, 0.61), intranasal (0.61 kg per day, 95% CI 0.57, 0.65), aerosol (0.64 kg per day, 95% CI 0.61, 0.68) when compared to the negative control group (0.73 kg per day, 95% CI 0.66, 0.80). Both intratracheal and intranasal groups differed in rate of gain versus the negative control group, but no difference was detected between aerosol and negative control groups (p > 0.05, Tukey-Kramer).

In general, mild coughing was noted on DPIs 3, 7, and 10 in pigs from the negative control group. Cough indices suggestive of respiratory compromise (> 2.5) were observed on 4 time points in the intratracheal group (DPEs 3, 7, 10, and 35), 8 in the intranasal group (DPEs 3, 14, 17, 24, 28, 31, 35, and 42), and 3 in the aerosol group (DPEs 3, 35, and 42). Overall, cough scores collected over the 49-day period were not significantly different among exposure groups (p > 0.05, Kruskall-Wallis).

3.2.2 Mycoplasma hyopneumoniae PCR

As was previously seen in the flow study, impinger samples collected during the pig exposure to aerosolized MHP were positive for MHP DNA and, with the exception of particles 0.8 µm in
size, *MHP* DNA (PCR) was detected in all particle sizes (1.6, 2.7, 4.0, 5.8, and >7.0 µm) collected with the Andersen Cascade Impactor.

Based on individual pig deep tracheal sample PCR testing, all animals in all 3 exposure groups were *MHP* DNA negative at -1 DPE and 70 of 72 *MHP*-exposed pigs were *MHP* DNA positive by 14 DPE (Figure 2). The distribution of *MHP* PCR Ct results by exposure route over DPE is shown in Figure 3. Based on 6 deep tracheal PCR results at 6 time points, the AUC analysis resulted in numerically higher shedding based on total PCR Cts in the intratracheal group (n = 648 PCR Cts; CI 95% 598 - 697), compared to intranasal (n = 573 PCR Cts; CI 95% 523 - 622), and aerosol (n = 572 PCR Cts; CI 95% 522 - 622), but no differences in the total *MHP* DNA among exposure routes were detected (p > 0.05, ANOVA). At necropsy, lung tissues from all *MHP*-exposed pigs were positive by PCR, with the exception of 1 pig (1/70) in the aerosol group. No differences in the LS means of lung tissue *MHP* PCR Ct values were observed among exposure routes (p > 0.05, ANOVA), i.e., intratracheal (25.0 PCR Ct, 95% CI 23.4, 26.6), intranasal (26.5 PCR Ct, 95% CI 24.9, 28.0), aerosol (24.2 PCR Ct, 95% CI 22.6, 25.7).

*MHP* DNA was detected in oral fluid samples from the intratracheal group at 7 time points (DPEs 15, 22, 25, 29, 32, 36, and 43), 5 in the intranasal group (DPEs 22, 29, 32, 36, and 43), and 8 in the aerosol group (DPEs 18, 22, 29, 32, 36, 39, 43, and 46). However, *MHP* DNA detection was inconsistent within exposure groups. That is, both pens were PCR positive in the intratracheal and aerosol groups on DPEs 36 and 43 and on DPEs 32 and 36 in the intranasal group. Overall, no difference in the oral fluid positivity rate over time was detected among exposure routes (p > 0.05, logistic mixed-effect regression). Overall, PCR Cts from oral fluid
samples were similar among MHP-exposed groups, i.e., intratracheal (mean = 33.52, sd = 1.77), intranasal (mean = 33.03, sd = 3.30), and aerosol (mean = 32.05, sd = 2.60).

3.2.3 *Mycoplasma hyopneumoniae* antibody response

*MHP* serum antibody was first detected in pigs (n = 4) from the intratracheal group at DPE 14 and 10 of 24 pigs (41.7%) were positive on DPE 21. *MHP* serum antibody was first detected in the aerosol (n = 1) and intranasal groups (n = 1) on DPE 21 (Figure 2), with 9 of 24 (37.5%) pigs in each group positive on DPE 28. At the termination of the study, 69/70 *MHP*-exposed pigs were *MHP* antibody positive, exception being one pig in the aerosol group. The distribution of *MHP* ELISA S/P ratios over 6 time points is shown in Figure 4. Significantly higher LS mean *MHP* ELISA S/Ps were observed in pigs intratracheally exposed to *MHP* compared to other groups (p < 0.05, Tukey-Kramer) at every DPE. No differences in ELISA S/Ps were detected between intranasal and aerosol groups.

3.2.4 Pathologic evaluation

At necropsy, no gross lung lesions were observed in the negative control group. Overall, the median gross lesion score in *MHP*-exposed pigs from the intratracheal group was 2.55% (min = 0%, max = 27%), intranasal 1.73% (min = 0%, max = 42.8%), and aerosol 1.59% (min = 0%, max = 30.5%), with no statistical difference among exposure groups (p > 0.05, Kruskall-Wallis). Based on the degree of hyperplasia, lungs from five negative control pigs were classified as unaffected and one was assigned a score of 4. The median degree of hyperplasia in lungs of *MHP*-exposed pigs was 4, with no significant difference detected among *MHP*-exposed groups (p > 0.05, Kruskall-Wallis). No IHC labeling was detected in lung tissues of pigs in the negative
control group or in 14 of the MHP-exposed pigs. IHC labeling was detected in 17 pigs in the intratracheal group, 19 pigs in the intranasal group, and 20 pigs in the aerosol. The overall median IHC score of 2 did not differ among exposure routes (p > 0.05, Kruskall-Wallis).

**Discussion**

*M. hyopneumoniae* remains one of the most challenging pathogens to control and eliminate in swine production systems. Notably, the majority of commercially available MHP vaccines are inactivated or adjuvanted whole-cell bacterins that provide partial protection (Maes et al., 2018), i.e., do not stimulate a robust and prolonged protective immunity. It follows that MHP control and elimination efforts are hampered by the absence of vaccines able to stimulate sterilizing immunity, long-term MHP infections, and the slow rate of transmission in partially immunized populations. For these reasons, most herd elimination and control protocols rely on the timely exposure of replacement gilts and sows to infectious MHP, thereafter allowing time for the animals' immune system to respond and clear the infection (Holst et al., 2016). This strategy has previously been used to control other pathogens in swine production systems, i.e., PRRSV (Desrosiers and Boutin, 2002), porcine epidemic diarrhea virus (Langel et al., 2016), and *Glaesserella parasuis* (Oliveira et al., 2004). Although producers commonly use intratracheal (Robbins et al., 2019) or aerosol (Yeske, 2017) routes of MHP exposure using MHP lung homogenate, the effect of route on the likelihood of achieving MHP infection and the animal's response to infection have not been evaluated.

Routes of MHP exposure were previously compared for their ability to induce mycoplasmal pneumonia under experimental settings (Garcia-Morante et al., 2016). The routes of MHP
exposure evaluated in this study are compatible with both research or commercial swine production settings, and were compared in terms of detectable parameters of infection (DNA and antibody), productivity (changes in average weight gain), and clinical signs (coughing and pulmonary lesions). Intratracheal MHP inoculation using low-cost medical feeding tubes is the standard in research settings (Pieters et al., 2009; Roos et al., 2016) and has likewise been used under field conditions (Robbins et al., 2019, Sponheim et al., 2020). Intranasal exposure using a laryngo-tracheal mucosal atomization device is used in humans to administer upper airway anesthetics (Fried et al., 2020) and similar type of device was used to inoculate pigs with cultures of MHP (Garcia-Morante et al., 2016). In this study, the mucosal atomization device has long, flexible canula small enough for complete introduction into the nares of pigs as young as 6 weeks of age and a tip that atomized the liquid to facilitate delivery of infectious material to MHP colonization sites. Cold foggers, such as the device used in this study, are used to apply disinfectants in farm facilities (Dee et al., 2005), mosquito pesticides (Williams et al., 2014), and poultry vaccines (Mohamed et al., 2016). More recently, their use in aerosolizing farm-specific MHP lung homogenate to animals during the course of the gilt acclimation has been reported (Yeske, 2017; Figueras Gourgues et al., 2020).

Regardless of the exposure method, delivery of viable MHP to the ciliated epithelium of the trachea and bronchi is fundamental to producing infection (Seymour et al., 2012). According to the manufacturer, the laryngo-tracheal mucosal device used for intranasal inoculation will generate particles ranging from 30 to 100 μm in diameter. Based on MHP DNA detection in deep tracheal samples from 18 of 24 pigs on DPI 7 and MHP serum antibody responses in 9 of 24 pigs in the intranasal exposure group on DPI 28, the device was shown capable of delivering
infectious particles to the desired site of *MHP* infection. Consistent with these results, Martini et al., (2020) found that a similar intranasal device generated droplet particles 86 μm (SD = 10 μm) in diameter and found that ~30% of the particles were deposited in the trachea and ~30% in the bronchi and lungs.

In the case of aerosol exposure, flow rate determines the distance and time of particle transport in the air (Asgharian et al., 2016). Evaluation of the three flow rates available with the commercial fogger using lung homogenate showed that the medium flow rate generated a significantly higher number of particles per m³ of air compared to the low flow rate and generated an aerosol over a longer period of time (9 min vs. 4.5 min) compared to the high flow rate. Thus, the medium flow rate was selected based on the higher likelihood of infection in exposed pigs. Although the size of aerosolized particles is dynamically affected by environmental temperature and relative humidity (Gralton et al., 2010), direct measurement of the number and size of particles per m³ of air during the process of exposure (Figure 1) showed the consistent presence of particles (“steady state”) ranging in size from 0.3 - 10 μm in diameter over the course of aerosolization. Recovery (6-Stage Andersen Cascade Impactor) and analysis (culture and PCR) demonstrated that the particles carried both infectious *MHP* and DNA. Thus, the commercial fogger was able to produce particles carrying infectious *MHP* and of a size able to reach the site of replication in the respiratory system, as demonstrated by *MHP* DNA detection in deep tracheal samples from 21 of 24 pigs in the aerosol group on DPE 7. During the aerosol exposure phase, a lower number of airborne particles per m³ of air was achieved as compared to the aerosol study using the same flow rate. This can be attributed both to reduced air flow due to the composition of the
inoculum, i.e., the presence of tissue debris and the viscosity of the Friis medium, and aerosolization in a larger air space (90.1 m$^3$ vs 180.35 m$^3$).

The course of infection differed somewhat by route of exposure. That is, numerical, but not statistical differences ($p > 0.05$), were observed in the proportion of deep tracheal swab $MHP$ DNA positive pigs on DPE 7, i.e., 24 of 24 intratracheal pigs, 18 of 24 intranasal pigs, and 21 of 24 aerosol of the pigs, but all pigs were $MHP$ DNA positive by DPE 21 and a comparison of PCR Ct values of positive pigs found no difference by route of exposure. Routes of exposure differed significantly ($p \leq 0.05$) in $MHP$ serum antibody responses, with earlier and higher ELISA S/P responses observed in the intratracheal group (Figure 2). Notably, one animal from the aerosol group demonstrated no infection, i.e., lack of detection of $MHP$ by PCR and IHC on lung tissues and no antibody response. Sill, this pig had continuously PCR positive results in deep tracheal samples, highlighting the complex biology of $MHP$, where potential colonization of the lower respiratory tract did not guarantee an infection sufficient to trigger a humoral immune response. Overall, the earlier detection of $MHP$ DNA and serum antibody suggested that direct deposition of $MHP$ into the trachea provided a more substantial exposure as opposed to intranasal and aerosol exposures.

Clinical signs of $MHP$ infection were observed in all exposure groups, but no differences in cough indices were detected among groups. Coughing events were numerically more frequent noted in intranasally exposed pigs ($n = 8$) versus intratracheal ($n = 4$) and aerosol ($n = 3$) exposures and it may be speculated the infection and inflammatory response in the upper respiratory tract caused irritation that stimulated coughing. Measurements of weight at two time
points (DPEs 28 and 49) showed differences in the rate of gain ($p \leq 0.05$) between the negative control group and intratracheal and intranasal groups, but no difference between the negative control and aerosol exposure group ($p > 0.05$). One pig from the negative control group scored 4 in the H&E assessment, but PCR and IHC negative in lung tissues. Pulmonary lesions associated with hyperplasia and presence of immune cells surrounding airways may occur occasionally in non-*MHP* inoculated animals, as has been reported in other *MHP* inoculation trials (Vicca et al., 2003; Oppriessing et al., 2004; Wooley et al., 2012). Albeit, examination of lung tissue collected at necropsy revealed no differences in lesion score among *MHP*-exposed groups. Interestingly, the 7 week timepoint post exposure is considerably later than the 4-week industry standard for assessing peak lesions in experimental infections (Thacker et al., 1999), which might indicate persistence of *MHP* lung lesions. Future studies should compare the longitudinal effects on weight gain, clinical signs, bacterial shedding, and lung lesion resolution. If any method can be cleared faster or have less clinical effect while still establishing infection, it may be advantageous for use in gilt acclimation.

An important part of gilt acclimation and *MHP* elimination programs is the confirmation of successful infection. Given the variability in *MHP* DNA and serum antibody detection in among pigs inoculated by different routes of exposure (Garcia-Morante et al., 2016), pigs were observed in this study for 49 DPEs to verify infection and seroconversion. PCR testing of deep tracheal samples offered the earlier (DPE 7 with intratracheal exposure) *MHP* DNA detection than oral fluids (DPE 15 with intratracheal exposure). While oral fluid samples revealed later and varied detection within exposure groups, at later time points post exposure (DPE 35), these samplings appeared to support confirmation, but studies to understand the performance of *MHP* PCR
protocols for oral fluid samples are needed. *MHP* serum antibody assayed with ELISAs provided conclusive confirmation, but later detection (DPE 21 with intratracheal exposure). In commercial swine settings, practitioners could benefit from this timely detection using DNA testing in deep tracheal samples to determine the need for re-exposure early on in the process. However, the cost per PCR test is significantly higher and deep tracheal sampling is more labour intensive compared to serum collection and antibody testing. Accordingly, practitioners may balance the cost of the sampling and the need for opportune determination of exposure.

Thus, all routes were ultimately successful in producing infection, with differences primarily observed in the time to the detection of DNA or serum antibody and minor clinical differences. In the field, specific production circumstances and the objectives of exposure, i.e. gilt acclimation or *MHP* elimination from an entire farm or system, will drive the selection of exposure route. Intratracheal inoculation is a reliable controlled exposure technique employed by most *MHP* research groups and some U.S commercial systems. Intranasal exposure devices are less invasive, but nevertheless require handling of individual pigs. Aerosol exposure is attractive because of its convenience, but likewise raises ethical and legal implications resulting from unintended exposure of animals and people to infectious airborne pathogens. Therefore, biosafety and biosecurity measures are paramount for this practice and must be taken into consideration. Notably, aerosol exposure needs to be done in relatively confined spaces with minimal airflow to provide sufficient exposure to the target population and simultaneously avoid the unintended exposure of other swine on the production site or neighboring sites, i.e., *MHP* DNA in air samples has been documented up to 4.7 km from a source of *MHP*-positive pigs (Dee et al., 2009). Personnel should use personal protective equipment throughout the process,
inoculum should be evaluated for purity using proper diagnostic testing based on the target microorganism (PCR, culture, etc), facilities should be thoroughly ventilated afterward, and proximity to other populations of swine should be taken into consideration.

**Conclusion**

Verification of infection was achieved in all treatment groups by 21 days post exposure, and allowed for the timely and uniformed infection required to initiate herd closure (day zero of infection) during pathogen elimination efforts and for gilt exposure in programs of acclimation. The three exposure routes performed similarly in terms of their ability to infect pigs with *MHP*. Intratracheal exposure resulted in earlier and higher antibody response measured with ELISA, while aerosol exposure had the least impact on average daily weight gain. Regardless, no difference was observed in coughing and no differences in lung lesions were detected at necropsy among the three routes. Intratracheal exposure is the current standard in research, but intranasal and aerosol exposure could be viable alternatives. Importantly, all routes of exposure require attention to the purity and quality of the inoculum. That is, inocula based on homogenized *MHP*-infected lungs can be contaminated with other infectious agents (Robbins et al., 2019; Figueras Gourgues et al., 2020). Thus, testing the inoculum for other viral and bacterial swine pathogens and potential zoonotic agents (i.e., influenza A virus, *Streptococcus* spp.) prior to use and compliance with strict biosafety and biosecurity practices are strongly recommended.
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Conflict of Interest

No conflict of interest is declared.

References


Tables

**Table 1**: Effect of fogger flow rates on aerosolization parameters using *Mycoplasma hyopneumoniae* (*MHP*) lung homogenate mixture

<table>
<thead>
<tr>
<th>Flow Rate</th>
<th>Time</th>
<th>Final Flow Rate</th>
<th>Air particles</th>
<th>6-stage Impactor</th>
<th>Impinger</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>19 min</td>
<td>52 mL per min</td>
<td>$8.27 \times 10^5$ (^a)</td>
<td>2 of 3</td>
<td>2 of 3</td>
</tr>
<tr>
<td>Medium</td>
<td>09 min</td>
<td>111 mL per min</td>
<td>$9.39 \times 10^5$ (^b)</td>
<td>3 of 3</td>
<td>3 of 3</td>
</tr>
<tr>
<td>High</td>
<td>4.5 min</td>
<td>222 mL per min</td>
<td>$9.43 \times 10^5$ (^b)</td>
<td>3 of 3</td>
<td>3 of 3</td>
</tr>
</tbody>
</table>

1 Fogger (Dyna-Fog® Hurricane "Cold Fog" ULV/Mister model 2792, Westfield, IN USA) operated at 3 flow rates of 189 mL per min (low), 236 mL per min (medium), and 266 mL per min (high). Results were based on 3 replicas at each flow rate (“fogging event”).

2 Time (mean) to deliver 1 L of *MHP* lung homogenate mixture (10 mL *MHP* lung homogenate in 900 mL commercial Friis broth medium with final concentration of $1 \times 10^5$ *MHP* strain 232 per mL).

3 The final flow rate of the device was calculated as the volume of *MHP* lung homogenate aerosolized over the time of aerosolization for each of the meter value settings (low, medium, high).

4 *MHP* Airborne particles (geometric mean) per m\(^3\) measured with an optical particle counter (AeroTrak™ Handheld Particle Sizer Model 9306-04, TSI Incorporated, Shoreview, MN USA). Different superscripted letters (“a” or “b”) indicate statistical differences ($p \leq 0.05$, linear mixed regression and Tukey-Kramer pairwise comparison).

5 Number of replicas of air samples with *MHP* PCR and culture positive results per flow rate ($n = 3$ replicas per flow rate) that were collected using a 6-stage Andersen Cascade Impactor.
(Series 10-800, Thermo Fischer Scientific Inc, Franklin, MA USA), operated throughout the fogging events. Stages of air particles 1.6, 2.7, 4.0, 5.8, and >7.0 µm in diameter were MHP PCR and culture positive results. While no MHP DNA was detected in particle of size 0.8 µm in diameter from any of 3 replicates using low, medium, high flow rate.

Number of replicas of air samples with MHP PCR and culture positive results per flow rate (n = 3 replicas per flow rate) that were collected using a SKC BioSampler® impinger (SKC Inc., Eighty Four, PA USA), operated throughout the fogging events.

Figures

Figure 1. Temporal distribution of Mycoplasma hyopneumoniae (MHP) airborne particles per m³ by particle size. Airborne particles were generated with a fogger (Dyna-Fog® Hurricane "Cold Fog" ULV/Mister model 2792, Westfield, IN USA) and were delivered at flow rates of 52 mL per min (low), 111 mL per min (medium), and 222 mL per min (high). Airborne particles per m³ of air measured with an optical particle counter (AeroTrak™ Handheld Particle Sizer Model 9306-04, TSI Incorporated, Shoreview, MN USA).

Figure 2. Pattern of Mycoplasma hyopneumoniae (MHP) DNA and antibody detection by route of exposure over a 42 and 49 day post-exposure (DPE), respectively, observation period. The inoculum (1 × 10⁵ MHP strain 232 per mL) was administrated intratracheally (n = 24 animals) via a feeding tube/urethral catheter (Integral Funnel, Two Eyes, Rounded Closed Tip, 4.7 mm x 41 cm, COVIDEN™ Kendall™, Coviden Ilc, Mansfield, MA USA), intranasally (n = 24 animals) using a laryngo-tracheal mucosal atomization device (MADgic®, Teleflex, Morrisville, NC USA), or via aerosol (n = 24 animals) using a fogger (Dyna-Fog® Hurricane "Cold Fog" ULV/Mister model 2792, Westfield, IN USA).

Figure 3. Detection of Mycoplasma hyopneumoniae (MHP) DNA in deep tracheal samples by route of exposure and day post exposure (DPE).

Figure 4. Antibody response to Mycoplasma hyopneumoniae (MHP) expressed as sample-to-positive ratios (S/P) by route of exposure (M hyo Ab Test, IDEXX Laboratories Inc.,
Westbrook, ME USA). Serum samples with ELISA S/P ≥ 0.30 (dashed line) were considered positive.