RESEARCH REPORT
SWINE HEALTH

Title: Gilt acclimation strategies for *Mycoplasma hyopneumoniae* control: Evaluation of exposure protocols - **NPB #18-116**

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Industry Summary:

A challenging aspect of achieving *M. hyopneumoniae* (*MHP*) disease control is the acclimation of incoming negative gilts into endemically infected farms. This is, in part, due to to the slow transmission rate of MHP that hinders timely exposure of naïve gilts when relying on pig-to-pig contact in gilt development units. Early *MHP* intentional exposure (before 80 days of age) to *MHP* during the acclimatization phase allows gilts to develop immunity and reduce shedding before their first farrowing. The objective of this study was to compare the efficacy of intratracheal, intranasal and aerosol MHP inoculation protocols of lung homogenate based on clinical signs, agent shedding, antibody response, and pulmonary lesions. Six-week-old gilts (n = 78) were randomized by weight to 4 groups: 1) aerosol exposure (cold fogger, n = 24), 2) intranasal exposure (atomization mucosal device, n = 24), 3) intratracheal exposure (intratracheal tube, n = 24), and 4) negative control (Friis media intratracheally, n = 6). The *MHP* inoculum consisted of lung homogenate (10^5 CFU/mL *M. hyo* strain 232). Prior to the aerosol exposure, a trial was carried out to determine the ideal flow rate used with the fogger. Weight was taken at -1, 28, and 49 day post-exposure (DPE). Serum and tracheal samples for antibody and DNA testing were collected weekly through DPE 49. Animals were humanely euthanized at 49 DPE. Lungs were evaluated for gross lesions and histopathology (H&E and IHC). All three routes succeeded in *MHP* infection, measured by PCR, ELISA and histopathology. Intratracheal exposure resulted in earliest detection of *MHP* DNA (7 DPE) and seroconversion (14 DPE), while intranasal and aerosol exposures showed

similar but delayed times for *MHP* DNA and antibody detection (14 DPE and 21 DPE). No significant differences in bacterial shedding (i.e. in deep tracheal swabs) was observed among exposure groups (total amount of *MHP* DNA). However, lower values of ELISA S/Ps with latter detection were observed in intranasal and aerosol when compared to the intratracheal exposure. Aerosol exposure had the least impact on daily weight gain (0.64 kg/day) compared to intranasal (0.61 kg/day) and intratracheal (0.58 kg/day), performing similarly to the negative control group (0.73 kg/day). At 49 DPE, gross and histopathologic lesions were observed in the lungs of 17/22, 19/24 and 19/24 gilts from the intratracheal, intranasal and aerosol exposure, respectively. In sum, specific production circumstances may affect the choice of *MHP* exposure route. For example, intranasal devices can be utilized to exposed weaned gilts, requiring less pig handling and time compared to the intratracheal route. Aerosol exposure offers the ability to expose larger groups of gilts, requires no pig handling, but can be challenging to achieve consistently in open or large facilities. Serology and tracheal sampling, depending on timing, vaccination and flow limitations will be key to measure level of exposure in acclimated gilts. Practical, safe and consistent gilt exposure methods will result in improved control and elimination programs for *MHP*.

Key Findings (3 bullet points):

1. Aerosol generated by the cold mist foggers at the medium flow rate carried live *M. hyopneumoniae* that is attached to the adequate airborne particle size (3-5 microns needed for successful infection.
2. Under the conditions of this study intranasal and aerosol exposure routes, which are less invasive and laborious compared to the intratracheal route, resulted in similar performance, and can be considered effective alternatives for safe acclimation of incoming naïve gilts.
3. A combination of serology and tracheal sampling will be critical for accurate and timely confirmation of successful *MHP* exposure.

Keywords: mycoplasmal pneumonia, gilt acclimation, diagnostics, aerosol exposure, fogging, intratracheal exposure, intranasal exposure.

Scientific Abstract (one page):

Mycoplasma hyopneumoniae (*MHP*) causes a chronic respiratory infection that significantly affects animal well-being, lowers feed conversion and predisposes pigs to co-infections. Intentional exposure to *MHP* during acclimation is intended to provide sufficient time for gilts to develop protective immunity and decrease *MHP* shedding before entering the breeding herd. The goal of this study was to compare the efficacy of intratracheal, intranasal and aerosol *MHP* inoculation protocols of live lung homogenate, in terms of clinical signs, *MHP* shedding, antibody response, and lung lesions. Six-week-old *MHP*- and PRRSV-negative gilts (n = 78) were

randomized to one of four *MHP* exposure groups [aerosol (n = 24); intranasal (n = 24); intratracheal (n = 24); or no exposure (n = 6)] and followed through 49 days post exposure (DPE). The *MHP* inoculum consisted of lung homogenate (105 CFU/mL MHP strain 232). All routes of exposure resulted in infection of gilts. Intratracheal demonstrated earlier detection (7 DPE), and seroconversion (14 DPE); intranasal and aerosol showed similar time to DNA and antibody detection (14 DPE and 21 DPE). PCR Ct values were similar in *MHP*-exposed groups over DPE ($p>0.05$), but lower ELISA S/Ps were observed in intranasal and aerosol exposures compared to intratracheal exposure. Aerosol exposed pigs experienced the least impact on daily gain (0.64kg/day), performing similarly to the negative control group (0.73kg/day). At 49 DPE, gross and histopathologic lesions were observed in the lungs of 17/22, 19/24 and 19/24 gilts from the intratracheal, intranasal, and aerosol groups, respectively. While the intratracheal exposure route, frequently carried out in research and certain pig production settings, is highly effective in generating *MHP* infection, the intranasal and aerosol routes resulted in similar performance. These alternative routes offer less animal handling, they are less labor-intensive and invasive intratracheal inoculation. Practical and efficacious gilt exposure methods will improve MHP control programs.

Introduction:

Mycoplasma hyopneumoniae (*MHP*) is endemic in the US and causes respiratory disease in pigs, resulting in significant economic losses due to increased mortality, slower growth rate and poorer feed efficiency.[1,2] It has been recently reported that the cost of *MHP*-associated disease is variable and increases tremendously when in combination with other viral agents, to up to $10 per pig.[1] Control of *MHP* infection in pig populations is typically based on establishing sow herd immunity by means of effective gilt acclimation (i.e. deliberate infection of gilts at an early age), strategic medication and vaccination. The overarching goal of creating robust herd immunity is to minimize sow shedding of *MHP*.[3] However, shedding of *MHP* from infected pigs is quite long (~254 days).[4] Therefore, the goal of acclimating gilts to *MHP* is to allow them to become infected early in life so they can develop protective immunity (i.e. quit shedding) before being introduced into the sow farm. This reduces the number of positive piglets at weaning, which has been shown to be a predictor for *MHP* clinical disease. [5]

Since swine breeding stock companies have largely eliminated *MHP* from their multiplication system, there is a need to immunize negative incoming gilts to *MHP* before entering in *MHP* positive commercial sow farms to minimize *MHP* shedding and infection of suckling pigs. [6] However, existing commercial vaccines are not effective at preventing infection, or significantly reducing shedding and disease.[7] Therefore, practitioners have attempted to deliberately infect gilts with *MHP* by exposing them to naturally infected animals (i.e. actively coughing pigs) in continuous-flow gilt development units (GDUs).[8,9] Some swine production systems have

carried out intratracheal inoculations of farm-specific *MHP* lung homogenate for gilt exposure.[10,11] However, the process is complicated, requiring experienced personnel, is labor-intensive and stressful for the animals. More recently, swine practitioners have explored the use of foggers for aerosolization of farm-specific *MHP* lung homogenate into confined air spaces to infect naïve gilts with a high degree of success.[9] This latter strategy, while innovative and practical, has not been properly validated to determine optimal conditions for successful and repeatable application on farm, such as dose, timing of aerosolization, and properties of the aerosol (i.e. droplet size).[12] Furthermore, it is likely that not all farms will have the appropriate farm conditions (i.e. facilities, ventilation, layout, size, etc) to be able to successfully implement aerosol inoculation protocols. Thus, another potential, less-explored approach is intranasal inoculation. The challenge with this route of exposure is that the preferred colonization site for *MHP* is the trachea and the bronchi, and less likely to colonize the nasal cavity. Therefore, intranasal inoculation protocols should rely on devices that disperse *MHP* particles and deposit them in the appropriate colonization sites, as the animal inhales.[13] In this study we evaluated the use of a novel and malleable atomization device for intranasal inoculation of *MHP*. This device is used in human medicine for pediatric nasal and tracheal drug administration in children. If successful, this minimally invasive tool could be easily implemented at the farm level, requiring little training of farm personnel. It is therefore critical to compare acclimatization strategies and to evaluate which one of them can achieve the best levels of *MHP* infection, followed by immunity.

Objective:

- To compare the efficacy of intranasal, intranasal and aerosol inoculation protocols of *M. hyopneumoniae* lung homogenate as methods to safely expose naïve gilts.

Materials & Methods:

1. Experimental design and sample collection

Six-week-old *MHP*- and PRRS-negative gilts (n = 78) were randomized by weight to 4 exposure groups into separated rooms: 1) negative control (n = 6); 2) aerosol exposure (n = 24); 3) intranasal exposure (n = 24); and 4) intratracheal exposure (n = 24). Sample size (24 pigs per inoculum) was based on the expectation of detecting a significant difference between proportions assuming 95% confidence level, 80% power, one-tailed test, in which 99% of pigs by intratracheal exposure, and 70% of pigs would become infected via aerosol or intranasal exposures.[15]

The inoculum used consisted of 10 mL lung tissue homogenate with 1×10^5 CCU/mL *MHP* strain 232 (Lot 44-2, Veterinary Diagnostic Laboratory, Iowa State University, ISU-VDL) from *MHP*-inoculated CDCD pigs diluted in 1:100 Friis broth mediumto a concentration estimated at 1.0×10^5 CCU/mL by titration.[14] Animals were observed daily for general health , and cough scores were collected at 3-to-4 day intervals (Cough Index Calculator (Boehringer Ingelheim Animal Health USA, Inc., Duluth, GA USA). Individual body weights were taken on -1, 28, and 49 days post exposure (DPE). Samples for antibody and DNA testing included serum and tracheal samples, and were collected on a weekly basis. Oral fluids and environmental aerosol samples (from negative and aerosol exposure groups) collected at 3-to-4 day intervals. Pigs were humanely euthanized on 49 DPE. At necropsy, lungs were evaluated for gross and microscopic lesions. Aerosols generated with the cold fogger mister were characterized prior to animal exposure, in terms of amount, size and *MHP* concentration attached to air particles.

2. Aerosol characterization and determination of ideal flow rate

Prior to the animal inoculation trial a study was carried out with the objective of identifying the ideal flow rate to be used and characterizing the aerosol produced by the cold mist fogger. The fogger (HURRICANE Ultra™ Electric Portable Aerosol Applicator ULV / Mister, Jackson, GA USA) was tested using three airflow rates, high (84 mL/min), medium (127 mL/min), and low (253 mL/min) with 3 replicas for each airflow. Total number of airborne particle was identified using optical particle counter (OPC) (AeroTrak 9306 Handheld Particle Sizer, TSI Inc. St. Paul, MN), classifying air particle size 0.1, 0.3, 0.5, 1.0, 5.0 and 10 µm in diameter. Linear mixed model tested differences of OPC (air particles/m^3) between airflow rates. The medium airflow rate (127mL per min) resulted in the highest amount of air particles, ($p < 0.05$) with more stability over time, compared to the low and high flow rates. Therefore, the medium flow rate was selected for the animal trial.

MHP concentration and viability were estimated with two aerosol collectors over 15 min (1) 6-stage Andersen Cascade impactor (ACI) (Series 10-800, Thermo Fischer Scientific Inc, Franklin, MA USA) and (2) SKC BioSampler® impinger (SKC Inc., Eighty Four, PA USA) for each replicate. ACI sampled air (28.3 L/min) and separated particles into 6 sizes (stages): > 7 µm (stage 1), 4.7-7 µm (stage 2), 3.3-4.7 µm (stage 3), 2.1-3.3 µm (stage 4), 1.1-2.1 µm (stage 5), 0.6-1.1 µm (stage 6); each particle size impacted on one agar plate (Friis medium agar-plate). The SKC BioSampler® (12.5 L/min) was loaded with 20 mL of Friis medium broth and airborne particles were recovered through impingement. Air samples of both aerosol collectors were tested for viability using culture (*MHP* titration using CCU method) and for concentration using PCR (MagMAXTM-96 Pathogen RNA/DNA extraction kit, Applied Biosystems™, Carlsbad, CA USA; and TaqMan® Fast Virus 1-Step Master Mix, Life Technologies, Carlsbad, CA USA, with primers and probes described for *MHP*183).

3. *M. hyopneumoniae* inoculation

Intratracheal exposure 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™ KendallTM, Coviden Ilc, Mansfield, MA USA) into the larynx, and then inoculating the lung homogenate (10 mL). Intranasal exposure was performed by inoculating the lung homogenate (10 mL per nostril) using a mucosal atomization device (MADgic®, Teleflex, Morrisville, NC USA) attached to a syringe. Administration of the intranasal inoculum was timed to coincide with pig inhalation to ensure deposition of *MHP* in the tissues required for colonization. Aerosol exposure was achieved by placing *MHP* lung homogenate (20 mL) and commercial Friis broth medium (Friis Media Broth, Teknova Inc., Hollister, CA USA) in the fogger which aerosolized at 127 mL/min for 18 min. The fogger was placed 1.5 m above the floor, directed toward the center of the room (6,500 m^3 area). Ventilation inlets were sealed to minimize loss of aerosol and the animals were stimulated to walk to increase breath rates.

4. Sample testing

MHP DNA in trachea, oral fluids, and lung tissue samples was extracted using MagMAXTM-96 Pathogen RNA/DNA kit (Applied Biosystems™) and amplified using TaqMan® Fast Virus 1-Step Master Mix (Life Technologies) with primers and probes described for *MHP*183. A sample was considered positive when Ct values were ≤ 40. Serum samples were tested using a commercial serum antibody ELISA (M hyo Ab Test, IDEXX Laboratories Inc., Westbrook, ME USA), following manufacturer's instructions, the antibody response was interpreted as $S/P < 0.3$ = negative, $0.3 \leq S/P \geq 0.4$ = suspect, and $S/P > 0.4$ = positive.

5. Pathology

At necropsy, lungs were evaluated and scored for gross lesions as described elsewhere (Halbur et al., 1995). Briefly, areas of consolidation (well-demarcated dark red-to-purple areas) were scored for total lung lesion given a 10% of contribution by right cranial lobe, right middle lobe, cranial part of the left cranial lobe, and the caudal part of the left cranial lobe, 5% by the accessory lobe, and 27.5% each by the right and left caudal lobes each. Lung tissue samples of normal and affected area were microscopically evaluated with hematoxylin and eosin (HE) and immunohistochemistry (IHC) staining.

6. Statistical analysis

The body weight response was analyzed for each pig using a linear mixed model, thereby, slopes and intercepts were compared across groups using ANOVA. Total *MHP* shedding by exposure route was compared using an area under curve (AUC) analysis. Thereafter, AUCs were compared among the 4 groups by ANOVA followed

by a Tukey-Kramer post-hoc test. Antibody response to *MHP* (ELISA S/P ratios) by exposure route was analyzed using linear mixed regression. Positive rate in oral fluids PCR testing was analyzed with mixed logistic regression. Differences in lung lesion scores between exposure routes (gross and microscopic lesions) and cough were assessed by using a non-parametric statistical method, Kruskall-Wallis test. Analyses were performed in R (R program version 3.6.0, R core team 2019).

Results:

The medium airflow rate (127mL per min) resulted in the highest amount of air particles, ($p < 0.05$) with more stability over time, compared to the low and high flow rates. Therefore, the medium flow rate was selected for the animal trial. *MHP* live colonies were attached to all examined air particles, as measured by the ACI and was also recovered from aerosol samples from the impinger, regardless of flow rate. A total of 1×10^7 *MHP* DNA copies were found to be attached to 10^8 air particles per m^3 of 3-5 microns, the size needed for adequate deposition in the airways, and thus, successful infection.

All three routes of exposure resulted in *MHP* infection, as evidenced by detection of *MHP* DNA and antibody response over time post exposure. Non-*MHP*-exposed gilts remained free of infection, i.e., negative *MHP* PCR in tracheal, oral fluid, and lung tissue samples and antibody in serum samples. Table 1 summarizes results by routes of *MHP* exposure in terms of DNA detection, antibody response, body weight, and lung lesions (grossly and microscopically). The 78 gilts weighted 26.6 kg (SD = 4.4; min = 17.5 kg; max = 39.7 kg) at -1 DPE. A significant effect on body weight gain was observed by comparing individual slopes among routes of exposure with the negative control group. Animals of intratracheal exposure group significantly had the lowest slope (0.58 kg/day) for body weight compared to aerosol exposure and negative control group ($p \leq 0.05$). However, intratracheal and intranasal (0.61 kg/day) exposures resulted in no differences of slopes ($p > 0.05$), whereas no difference was also observed between slopes of aerosol exposure (0.64 kg/day) and negative control (0.73 kg/day). No differences in coughing scores between exposure routes was observed in this study (Kruskall-Wallis test, $p > 0.05$). Cough indices generally resulted in low values for any *MHP* exposure, i.e. intratracheal exposure resulted in 1.64%, intranasal 2.3%, and aerosol 1.33%.

MHP infection was confirmed by PCR in trachea samples at 7 DPE in all groups (n = 69 of 72 *MHP*-exposed gilts). No differences in total DNA shedding was observed between exposure routes (AUC, ANOVA, $p > 0.05$) (Figure 1). DNA detection in pen-based oral fluids was inconsistent over DPE by exposure route with no differences in *MHP*-positivity rate (mixed logistic regression, $p < 0.05$). Antibody response was generally detected by 21DPE but seroconversion was observed significantly earlier in the intratracheal group(15 DPE)

with higher values of ELISA S/P ratios (0.504; CI 95% 0.428 – 0.580) as estimated by linear mixed regression ($p < 0.05$) (Figure 2). Animals exposed by aerosol and intranasal seroconverted at 22 DPE with statistical equivalence in terms of ELISA S/P ratios (0.281; CI 95% 0.204 – 0.357 and 0.329; CI 95% 0.252 – 0.405). At the termination of the study, 71 *MHP*-exposed gilts seroconverted, with exception of one animal from the aerosol group. Lung tissues from *MHP*-exposed animals were positive with PCR testing, with exception of one animal of aerosol exposure resulted in *MHP* DNA negative. No statistical differences in terms PCR Ct values, gross or histopathological , = lung lesions were observed in this study (Kruskall-Wallis test, $p > 0.05$).

Table 1. Comparison of *MHP* exposure routes.

	Intratracheal	Intranasal	Aerosol
Shedding (Traquea PCR)			
- time to 100% gilts positive	7 DPE	14 DPE	14 DPE
- Maximum MHP (Cts)	20.8	21.6	21.03
Antibody response (Serum ELISA)			
- time to detect fisrt positive	14 DPE	21 DPE	21 DPE
- 100% detection	42 DPE	49 DPE	>49 DPE
- mean S/P ratio[1]	0.504[a]	0.329[b]	0.281[b]
Weight gain (kg / day)[1,2]	0.58[a]	0.61[a,b]	0.64[b,c]
Lung lesions			
- Gross (median of scores)	2.55%	1.73%	1.59%
- HE[3]	21 / 22	24 / 24	21 / 24
- IHC[3]	17 / 22	19 / 24	20 / 24

[1]Different superscripted letter represent statistical difference ($p<0.05$).
[2]Control group: 0.73kg/day[c]
[3]Number of lungs with microscopic lesions per total of gilts.

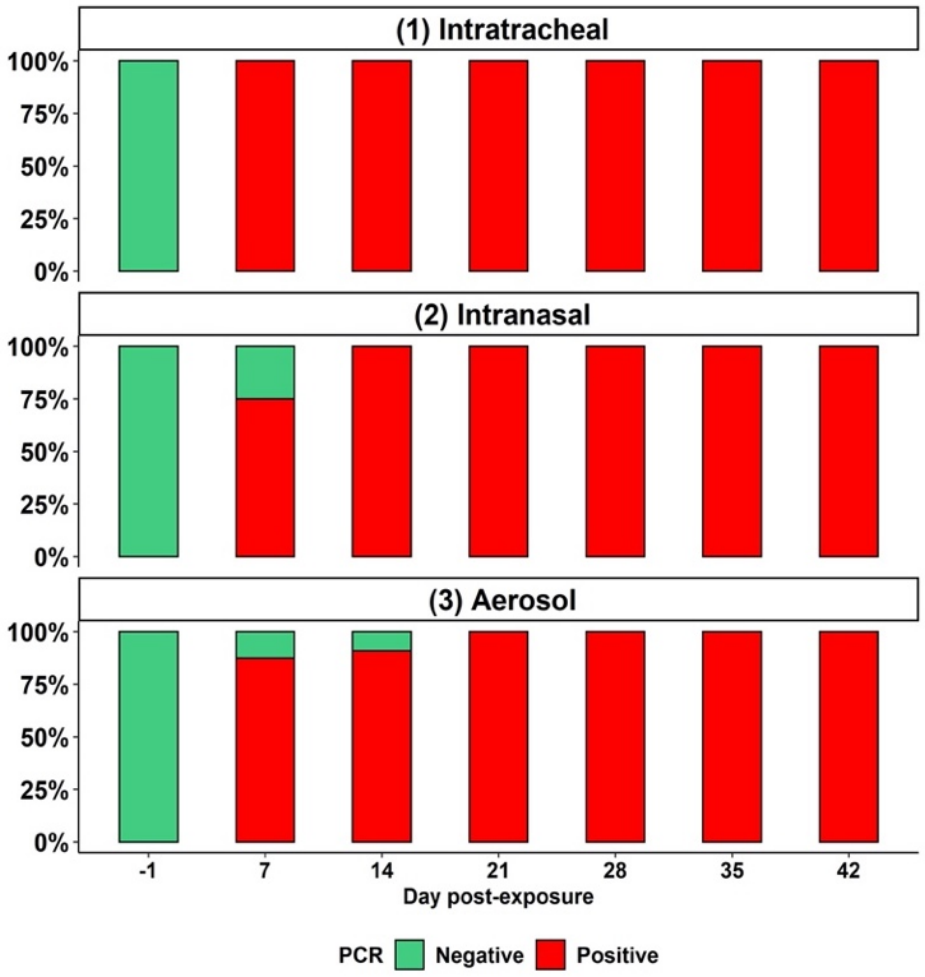

Figure 1. Proportion of *MHP* PCR positive tracheal samples by exposure route and DPE.

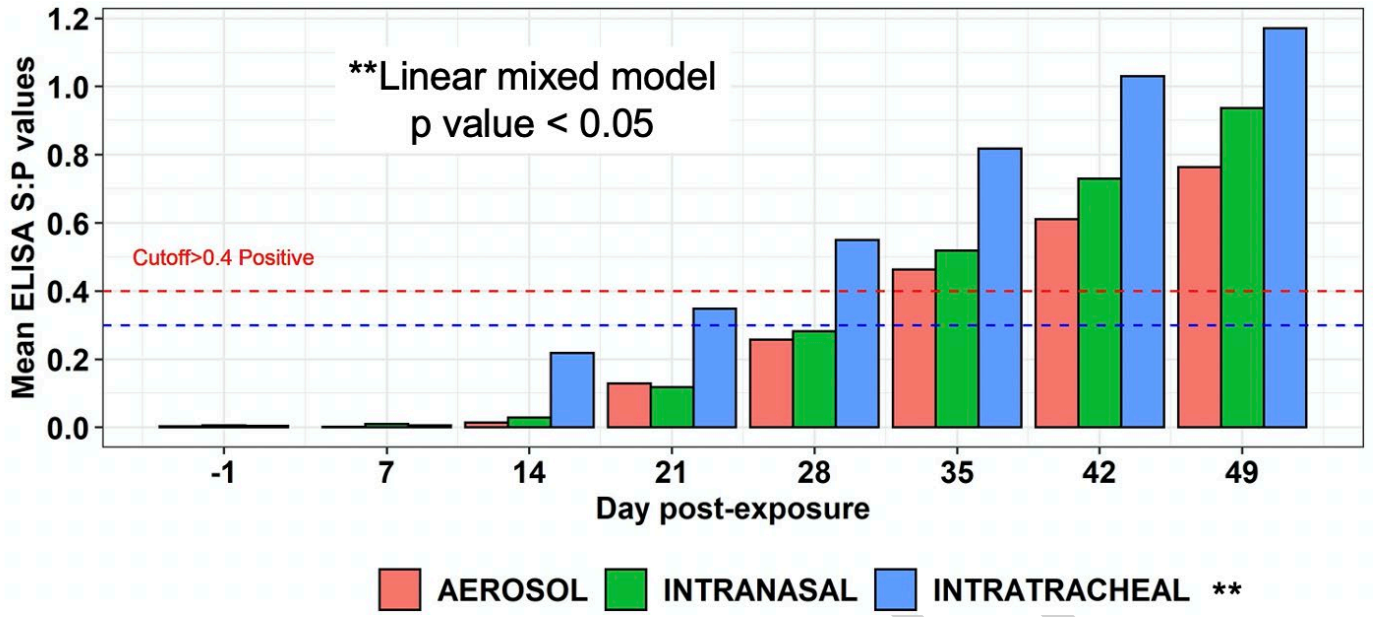

Figure 2. Antibody response to *Mycoplasma hyopneumoniae* (*MHP*) by routes of exposures in terms of ELISA sample-to-positive values (ELISA S/P ratios).

I. Discussion:

MHP significantly affects pig production and it is recognized as a main driver for antimicrobial use by swine farms. *MHP* control hinges upon effective and early gilt acclimation to ensure minimal transmission in the farrowing room and, thus, guarantee a reduction in clinical disease in the downstream pig flow. While vaccines are highly effective at reducing the clinical impact of the disease, they do not prevent infection and do not significantly decrease shedding. Therefore, gilt acclimation for *MHP* is based on either exposure of naïve gilts to positive animals in the GDU or the implementation of controlled exposure program to generate uniform exposure. In this study, we attempted to compare exposure routes that could be easily implemented under field conditions when scaled to fit larger pig populations.

In-depth characterization of the *MHP* lung homogenate aerosol produced by the fogger was previously unknown. The fogger set at 127 mL/min generated 10^7 *MHP* DNA copies per m^3 attached to air particles of 3-5µm, which is the size needed for trachea and bronchi colonization. Therefore, this study showed that live *MHP* is attached to specific airborne particles needed for successful infection.

All exposure routes resulted in *MHP* infection, development of immune protective response, and caused comparable detection, pathological lesions and similar body weight gain throughout 49 days of acclimatization. Specific production circumstances may affect the choice of exposure route. For example, intranasal devices can be utilized to exposed weaned gilts, requiring less pig handling and time compared to the intratracheal route. In contrast, aerosol exposure offers the ability to expose large groups of gilts, requires no pig handling, but can be challenging to achieve in opened and larger facilities. Serology and tracheal sampling, depending on timing, vaccination and flow limitations will be key to measure level of exposure in acclimated gilts. As previously reported, pen-based oral fluid samples performed poorly in this study to measure timely exposure to *MHP*.[16] As exemplified in this study, tracheal samples by PCR (~ 7 DPE) offered earliest *MHP* detection that practitioners can use to quickly confirm exposure and determine the need for re-expose of gilts. In contrast, while detection of serum antibodies by ELISA testing is less costly than PCR and it is an easier sample to collect, detection of widespread infection in a population takes longer (> 28 DPE). Under the conditions of this study intranasal and aerosol, which are more animal welfare friendly, resulted in similar performance as intratracheal exposure, and they can be considered effective alternatives for gilt acclimation. Practical and consistent gilt exposure methods will result in improved control and elimination programs.

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