Assessment of nebulization technology for gilt exposure to *Mycoplasma hyopneumoniae* as an acclimation strategy

Sebastián Figueras Gourgues, DVM, PhD; Eduardo Fano, DVM, PhD; Ana Alegre Sabaté, DVM; Elisa López Grasa, DVM; Iván Hernández Caravaca, DVM, PhD; Francisco A. García Vázquez, DVM, PhD; Víctor Rodríguez Vega, DVM; Beatriz Garcia-Morante, DVM, PhD

Summary

Objective: This study assessed the efficacy of nebulization (NEB), also known as fogging, to expose gilts to *Mycoplasma hyopneumoniae* under field conditions as a potential acclimation strategy.

Materials and methods: Phase I consisted of 448 *M hyopneumoniae*-free gilts from four different batches of a gilt development unit (GDU). On study day 0, batches 1 and 2 were exposed to *M hyopneumoniae*-positive lung homogenate via intratracheal (IT) route and were used as reference for batches 3 and 4, which were exposed using a mechanical fogger. Tracheobronchial swabs (TBS) were collected at 2 and 4 weeks post exposure (D14 and D28, respectively) and infection success was assessed by real-time polymerase chain reaction of pooled samples. In phase II, 1160 gilts from the same GDU belonging to three different batches (5 to 7) were exposed to *M hyopneumoniae* via NEB, and TBS were collected at D14.

Results: In phase I, no statistically significant differences were observed between IT and NEB exposure in proportion of positives and mean cycle threshold values of TBS pooled samples at any time point (D14 and D28). In phase II, TBS pooled samples from all batches were positive for *M hyopneumoniae* at D14.

Implications: Nebulization of lung homogenate positive for *M hyopneumoniae* resulted in infection of commercial gilts with this pathogen. Therefore, the use of NEB may be a reliable *M hyopneumoniae* exposure method under field conditions. The information generated in this investigation broadens the understanding of this technology as an acclimation strategy.

Keywords: swine, gilt acclimation, lung homogenate, *Mycoplasma hyopneumoniae*, nebulization

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Resumen - Evaluación de la tecnología de nebulización para la exposición de las primerizas a *Mycoplasma hyopneumoniae* como estrategia de aclimatación

Objetivo: Este estudio evaluó la eficacia de la nebulización (NEB), también conocida como fogueo, para exponer a las primerizas a *Mycoplasma hyopneumoniae* en condiciones de campo como una posible estrategia de aclimatación.

Materiales y métodos: La fase I consistió en 448 primerizas libres de *M hyopneumoniae* de cuatro lotes diferentes de una unidad de desarrollo de primerizas (GDU). El día 0 del estudio, los lotes 1 y 2 se expusieron a un homogeneizado de pulmón positivo a *M hyopneumoniae* por vía intratraqueal (IT) y se utilizaron como referencia para los lotes 3 y 4, que se expusieron utilizando un nebulizador mecánico. Se recogieron hisopos traqueobronquiales (TBS) en las 2 y 4 semanas posteriores a la exposición (D14 y D28, respectivamente) y se evaluó el éxito de la infección mediante la reacción en cadena de la polimerasa en tiempo real de muestras agrupadas. En la fase II, 1160 primerizas de la misma GDU pertenecientes a tres lotes diferentes (5 a 7) se expusieron a *M hyopneumoniae* a través de NEB, y se tomaron TBS en el D14.

Resultados: En la fase I, en ningún momento (D14 y D28) se observaron diferencias estadísticamente significativas entre la exposición IT y NEB en la proporción de positivos y de los valores de umbral de ciclo medio de las muestras agrupadas de TBS. En la fase II, en el D14, las muestras agrupadas de TBS de todos los lotes fueron positivas a *M hyopneumoniae*.

SFG, IHC, FAGV: Department of Physiology, Faculty of Veterinary Science, University of Murcia, Murcia, Spain.

EF: Boehringer Ingelheim Animal Health Inc, Duluth, Georgia.

BG-M: Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St Paul, Minnesota.

AAS, ELG: Piensos Costa, Fraga, Spain.

VRV: Department of Animal Health, Faculty of Veterinary Science, Complutense University of Madrid, Madrid, Spain.

Corresponding author: Dr Beatriz Garcia-Morante, Veterinary Population Medicine Department, College of Veterinary Medicine, University of Minnesota, 1365 Gortner Ave, St Paul, MN 55108; Tel: 612-990-8714; Email: bea.garciamorante@gmail.com.

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Implicaciones: La nebulización de homogeneizado de pulmón positivo a *M hyopneumoniae* resultó en la infección de primerizas comerciales con este patógeno. Por lo tanto, el uso de NEB puede ser un método confiable de exposición a *M hyopneumoniae* en condiciones de campo. La información generada en esta investigación amplía la comprensión de esta tecnología como estrategia de aclimatación.

Résumé – Évaluation d'une technologie de nébulisation pour l'exposition de cochettes à *Mycoplasma hyopneumoniae* comme stratégie d'acclimatation

Objectif: Cette étude a évalué l'efficacité de la nébulisation (NEB), également connue sous l'appellation brumisation, pour exposer des cochettes à *Mycoplasma hyopneumoniae* dans des conditions de terrain comme une stratégie potentielle d'acclimatation.

Matériels et méthodes: La phase I consistait en 448 cochettes exemptes de *M hyopneumoniae* provenant de quatre lots différents d'une unité de développement des cochettes (GDU). Au jour 0 de l'étude, les lots 1 et 2 furent exposés à un homogénat de poumon positif pour *M hyopneumoniae* via la voie intratrachéale (IT) et furent utilisés comme référence pour les lots 3 et 4, qui furent exposés à l'aide d'un nébuliseur mécanique. Des écouvillons trachéobronchiques (TBS) furent prélevés à 2 et 4 semaines post-exposition (D14 et D28, respectivement) et le succès de l'infection fut évalué par réaction d'amplification en chaîne par la polymérase en temps réel d'échantillons regroupés. Dans la phase II, 1160 cochettes provenant de la même GDU et appartenant à trois lots différents (5 à 7) furent exposées à *M hyopneumoniae* via NEB, et des TBS prélevés à D14.

Résultats: Dans la phase I, aucune différence statistiquement significative ne fut observée entre l'exposition IT et NEB en proportion de positifs et des valeurs moyennes de seuil de cycles des échantillons de TBS regroupés à n'importe quel point d'échantillonnage (D14 et D28). Dans la phase II les échantillons groupés de tous les lots étaient positifs pour *M hyopneumoniae* à D14.

Implications: La nébulisation d'un homogénat de poumon positif pour *M hyopneumoniae* a résulté en une infection de cochettes commerciales avec cet agent pathogène. Ainsi, l'utilisation de NEB pourrait être une méthode fiable d'exposition à *M hyopneumoniae* dans des conditions de terrain. L'information générée dans cette étude élargie la compréhension de cette technologie dans une stratégie d'acclimatation.

Mycoplasma hyopneumoniae is the etiologic agent of mycoplasmal pneumonia, a chronic bronchopneumonia which impacts swine health worldwide.[1] Infection with *M hyopneumoniae* predisposes pigs to infections with other respiratory bacteria and viruses, playing an important role in more clinically and economically relevant diseases known as enzootic pneumonia (EP) and the porcine respiratory disease complex (PRDC).[1] Despite all efforts implemented to reduce the economic impact attributed to *M hyopneumoniae*, EP and the PRDC are still associated with important economic losses to the swine industry.

Although indirect contact has importance in the infection dynamics of *M hyopneumoniae*, direct nose-to-nose contact between infected and susceptible pigs is considered the main route of transmission.[2] First exposure to *M hyopneumoniae* occurs during lactation when piglets may become infected in the farrowing unit through shedding sows.[3-5] Several studies have demonstrated that piglets may be colonized during the lactation period and are then positive with this bacterium when weaned.[6-9] Moreover, it has been shown that disease severity in growing pigs could be correlated with *M hyopneumoniae* piglet prevalence at weaning.[9,10]

Circulation of *M hyopneumoniae* is thought to occur among existing sows and be transmitted to incoming gilts.[3] An inverse relationship between parity number and *M hyopneumoniae* shedding has been described, thus, gilts and low parity sows infected for the first time are considered the main source of the bacteria to suckling piglets.[2,11,12] In addition, the existence of negative subpopulations that can reach 20% of the gilt population have been described in positive herds.[13] This, together with *M hyopneumoniae* shedding that can persist up to 214 days post infection,[14] make the implementation of an early and proper gilt acclimation process against *M hyopneumoniae* of paramount importance. Therefore, an adequate gilt acclimation pursues the elimination of bacterial shedding at first farrowing to minimize piglet colonization and later problems in the growing phase.[15,16]

Vaccination is the main strategy used for replacement gilt acclimation procedures against *M hyopneumoniae* in both Europe and North America.[16] Vaccination protects the gilts although it does not stop them from being infected and shedding the organism.[17,18] Another frequently used acclimation strategy is direct contact with pigs that are suspected to be infected.[19-21] In this strategy, a uniform infection with *M hyopneumoniae* is difficult to achieve as transmission is known to be very slow.[22,23] To increase the success of infection, the use of lung tissue homogenate containing *M hyopneumoniae* to deliberately infect replacement gilts has been recently reported in the United States and Mexico.[20,21,24,25]

Controlled exposure of naïve gilts to *M hyopneumoniae* infective material might be a complementary method for gilt acclimation that deserves further investigation. The intratracheal (IT) method is the most widely used in *M hyopneumoniae* experimental inoculation procedures.[26] However, due to the difficulty that this method represents at a large scale in the swine industry, this study assessed the efficacy of *M hyopneumoniae* exposure using nebulization (NEB), also referred to as fogging, under field conditions. Efficacy of exposure was determined by real-time polymerase chain reaction (qPCR) testing of pooled tracheobronchial swabs (TBS) collected at 2 and 4 weeks post exposure to confirm *M hyopneumoniae* infection.

Materials and methods

Animals and housing

The study was conducted from July 2017 to June 2018 in a 2200-head gilt development unit (GDU) located in Aragón, Spain. This farm consists of two barns, each 854 m^2; each barn had two 420 m^2 units. The gilts (Landrace × Large White crossbred) in the study were housed in 9 m^2 pens in groups of 10 within the same unit. The GDU worked in batches depending on sow farm demand and used an all-in/all-out management system. Feed and water were available *ad libitum* in stainless steel feeders and through water nipples, respectively. Gilts were raised in facilities with fully slatted floors and fed

a diet to meet or exceed their nutritional needs. All animals were under veterinary oversight and care with a veterinarian-client-patient relationship and a Welfare Quality based certification in place (animal welfare certification by Asociación Española de Normalización y Certificación). Gilts weighed approximately 20 kg upon entry to the GDU (approximately 7 weeks of age) and approximately 100 kg upon departure from the GDU (approximately 28 weeks of age). All gilts were obtained from a unique nucleus and multiplier pig farm known to be negative for *M hyopneumoniae*, wild type porcine reproductive and respiratory syndrome virus (PRRSV), and influenza A virus. The routine vaccination program included immunization against PRRSV, Aujeszky's disease virus, influenza A virus, porcine parvovirus, *Erysipelothrix rhusiopathiae*, *Actinobacillus pleuropneumoniae*, porcine circovirus type 2, and *M hyopneumoniae* as a growing pig (starting at 10-12 weeks of age) followed by a booster immunization 3 to 4 weeks apart. Prior to study initiation, no signs of any major pig diseases were observed. No antimicrobials were administrated to gilts under study.

Experimental design and sample collection

This research consisted of two phases: phase I had a total of 448 gilts from 4 batches (1-4) and phase II had 1160 gilts from 3 batches (5-7). The experimental design is presented in Table 1. In all cases, batches were formed with a varying number of gilts and exposure to *M hyopneumoniae* occurred at 10 to 13 weeks of age (study day 0). In phase I, batches 1 and 2 were used as positive controls and were inoculated via IT, while batches 3 and 4 were exposed to the pathogen by NEB. In phase II, all batches (5-7) were exposed to *M hyopneumoniae* via NEB at 10 to 13 weeks of age. In each batch, a subgroup of 30 gilts was randomly selected and monitored for infection confirmation during the acute phase of infection. For this purpose, TBS were collected at 2 and 4 weeks post exposure (D14 and D28, respectively) in phase I, and at D14 in phase II.

M hyopneumoniae infectious material

The infectious material for batches 1 and 2 was obtained from a commercial farrow-to-wean herd with EP problems in replacement gilts at entry and in offspring at finishing stages. The herd received gilts from the GDU used in this study. Ten, clinically affected, 24-week old gilts were selected and subjected to TBS sampling. The seed material donor was identified based on the minimum presence of other swine respiratory pathogens and the lowest cycle threshold (Ct) to *M hyopneumoniae* as determined by qPCR and described by Robbins et al.[27] Subsequently, the selected donor was euthanized, necropsied, and the lung tissue was used to prepare the seed tissue homogenate. For batches 3 and 4, seven gilts from the GDU were artificially inoculated with *M hyopneumoniae* (batch 1), euthanized, and necropsied. Seed material donors were selected by testing lung homogenate and using qPCR and the same criteria previously mentioned. In this case, lungs from 3 gilts were selected to proceed with the seed tissue homogenate preparation. Lastly, lungs from 10 gilts belonging to previous GDU batches exposed to *M hyopneumoniae* were used for the seed lung homogenate preparation for batches 5, 6, and 7. In all cases, seed tissue homogenates were prepared roughly as a ratio of 6 g of lung tissue for every 4 mL of homemade Friis medium. Thereafter, the homogenates were confirmed to be positive for *M hyopneumoniae* and stored in 30 mL aliquots at -80°C until used. Presence of *M hyopneumoniae* and other pathogens in the three seed lung homogenates are shown in Table 2.

M hyopneumoniae inoculation

Gilts from batches 1 and 2 (phase I) were inoculated once via IT with 10 mL of inoculum. The inoculum was prepared with the seed lung homogenate at a dilution of 1:50 in Friis medium and at a final concentration of 4.6×10^6 genome copies/mL, as determined by qPCR. The inoculation technique was performed as previously described by Pieters et al,[14] but without the use of anesthesia. Briefly, a post cervical insemination catheter (Magaplus; Magapor) was used for lung homogenate delivery into the trachea, and a laryngoscope and a mouth gag used for visualization. An electric portable aerosol applicator (Hurricane Ultra; Curtis Dyna-Fog Ltd) was used to expose gilts in batches 3 to 7 (phase I and II) to *M hyopneumoniae* via NEB. In this case, inoculum was prepared with the seed lung homogenate at a dilution of 1:50 in Friis medium and grossly filtered to discard tissue debris to avoid equipment malfunction. In phase I, inocula final concentrations of *M hyopneumoniae* were 6.6×10^6 and 8.9×10^6 genome copies/mL in batch 3 and 4, respectively. In phase II, inocula final concentrations were 1.1×10^4, 2.1×10^5, and 1.6×10^7 genome copies/mL in batch 5, 6, and 7, respectively. Infectious material was administered with a total output rate of approximately 236 mL/min at 220 volts over 2 minutes in each pen housing 10 gilts. The fogger was manually focused toward the gilts' snouts and a left-right movement made to ensure that all animals inhaled the aerosol. The particle sizes generated ranged from 7 to 30 μm of volume mean diameter depending on the flow rate and viscosity of the inoculum. During the NEB procedure, all barn windows were closed to avoid air flows that could interfere with the exposure of the gilts to the infectious material. Additionally, all personnel that

Table 1: Experimental design for gilts artificially exposed to *M hyopneumoniae* using two inoculation techniques

Study phase	Batch No.	No. of gilts	D0 Inoculation method	TBS*	
				D14	D28
I	1	88	IT	yes	yes
	2	120	IT	yes	yes
	3	120	NEB	yes	yes
	4	120	NEB	yes	yes
II	5	370	NEB	yes	no
	6	386	NEB	yes	no
	7	404	NEB	yes	no

* Thirty gilts within each batch were randomly selected for TBS sampling for *M hyopneumoniae* detection by qPCR.

TBS = tracheobronchial swabs; IT = intratracheal; NEB = nebulization; qPCR = real-time polymerase chain reaction.

Table 2: Presence of *M hyopneumoniae* and other pathogens in seed lung homogenates for gilt exposure

Pathogen tested	Ct values		
	Batches 1 and 2	Batches 3 and 4	Batches 5, 6, and 7
PRRSV-1*	Neg	32.89	Neg
PRRSV-2	Neg	Neg	Neg
Influenza A virus	Neg	Neg	Neg
Porcine circovirus type 2	Neg	Neg	Neg
M hyopneumoniae †	**23.82**	**23.90**	**20.99**
Mycoplasma hyorhinis	Neg	25.98	Neg
Actinobacillus pleuropneumoniae	Neg	Neg	33.46
Streptococcus suis	Neg	Neg	Neg
Pasteurella multocida	26.36	Neg	33.24
Haemophilus parasuis	Neg	Neg	Neg
Bordetella bronchiseptica	Neg	Neg	Neg

* Pathogen detected had > 98% homology with the vaccine PRRSV strain by comparing open reading frame 5 sequences.
† A sample was considered positive for *M hyopneumoniae* when the Ct value was ≤ 38.
Ct = cycle threshold; PRRSV = porcine reproductive and respiratory syndrome virus; Neg = negative.

could be putatively exposed to the aerosol used personal protection equipment including a respirator (3M 4279 Reusable Half Face Masks; 3M) and goggles (3M GoggleGear 500 Series GG501SGAF; 3M).

Sample collection, processing, and testing

Thirty gilts within each exposed batch were randomly selected for TBS sampling at D14 and D28, which were obtained as previously described by Fablet et al.[28] A gilt was restrained with a nose snare and a mouth gag and laryngoscope were used for visualization. A post cervical insemination catheter was used to reach the trachea-bronchial bifurcation where mucus was collected through gentle catheter movement. The tip of the catheter (2-cm diameter) was placed in a 5 mL BD Serum Vacutainer tube (Becton Dickinson and Company), mixed with 2 mL sterile saline, and refrigerated until testing. Individual TBS were tested in pools of 5. Each sample or sample pool was sent to EXOPOL S.L.U. (Zaragoza, Spain) and analyzed using an *M hyopneumoniae* specific qPCR (EXOone *M hyopneumoniae* oneMIX qPCR; EXOPOL S.L.U.), which has been validated using a DNA purification kit (UltraClean Tissue & Cells DNA Isolation Kit; MOBIO Lab, Inc) for DNA extraction. The qPCR kit contains an endogenous control to avoid false negative results and ensure that the entire process has been correctly performed. A sample was considered positive for *M hyopneumoniae* when the Ct value was ≤ 38.

Data analysis

Statistical analyses and data summaries were performed using Graph Pad Prism 8 software. All data were summarized descriptively based on the type of variable and analyzed assuming a completely random design structure. An analysis of variance through the ordinary one-way ANOVA was applied for mean comparison of qPCR Ct values among gilt batches at D14 and D28. The Chi square test was used to evaluate the proportion of positive qPCR samples between groups at different sampling points. Tests on differences were designed as 2-sided tests at $\alpha = .05$, with differences considered significant if $P \leq .05$.

Results

Phase I: Batches exposed via IT vs NEB

The Ct values of the positive TBS pooled samples in the acute phase of infection (D14 and D28) from batches 1 to 4 are shown in Figure 1. Overall, no statistically significant differences were observed in proportion of positive TBS pooled samples or in potential bacterial load (mean Ct value) between batches at any time point. At D14, all samples were positive for *M hyopneumoniae* in all batches, regardless of the exposure method (ie, IT or NEB). At D28, 4 of 6 (66.7%) TBS pooled samples were positive for *M hyopneumoniae* in batch 1, 5 of 6 (83.3%) samples were positive in batches 2 and 3, and 6 of 6 (100%) samples were positive in batch 4.

Phase II: Batches exposed via NEB

The Ct values of the positive TBS pools in the acute phase of infection (D14) from batches 5 to 7 are shown in Figure 2. No statistically significant differences were observed in proportion of positive TBS pools or in indicative bacterial load (mean Ct value) between batches at D14. In parallel to phase I, all pooled samples were positive for *M hyopneumoniae* in all batches at D14.

Discussion

Because gilts might be the major source of *M hyopneumoniae* to newborn pigs,[2,3] a suitable gilt acclimation focused on reducing the bacterial shedding at first farrowing has been suggested.[15] Information on gilt acclimation strategies for *M hyopneumoniae* is limited; a recent review has pointed out that vaccination is the main strategy used in Europe, Mexico, and the United States.[16] Vaccination of gilts for *M hyopneumoniae* at acclimation may be effective to decrease shedding and infectious pressure,[29] however, studies under experimental[18] and field conditions[7,30] showed that vaccination did not prevent infection and transmission of

Figure 1: Tracheobronchial swab (TBS) sampling two (D14) and four (D28) weeks post exposure to *M hyopneumoniae* using two inoculation methods. Individual and mean (SD) Ct values of positive TBS pooled samples using a qPCR test for *M hyopneumoniae*. Ct = cycle threshold; qPCR = real-time polymerase chain reaction; IT = intratracheal; NEB = nebulization.

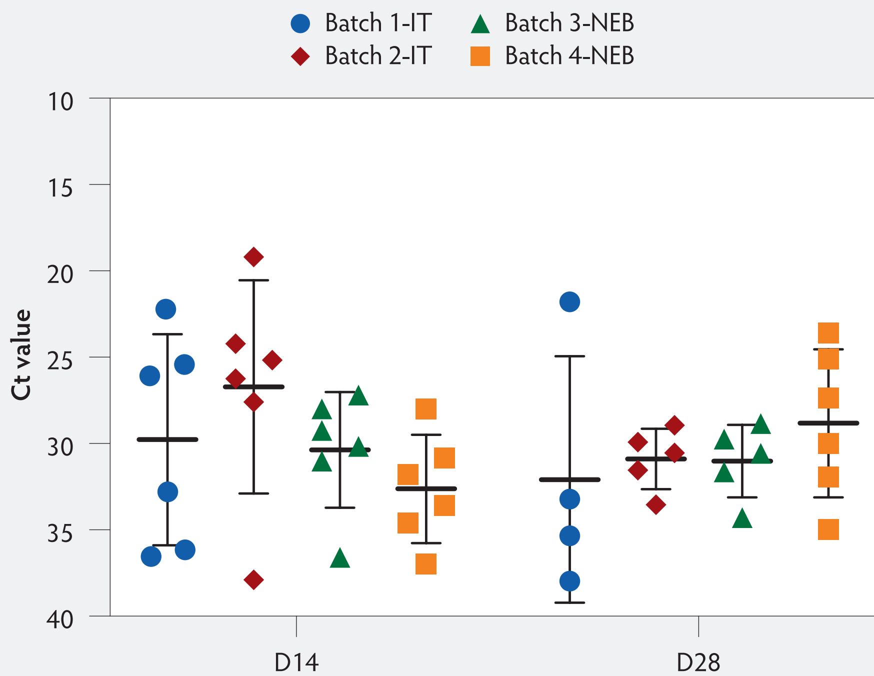
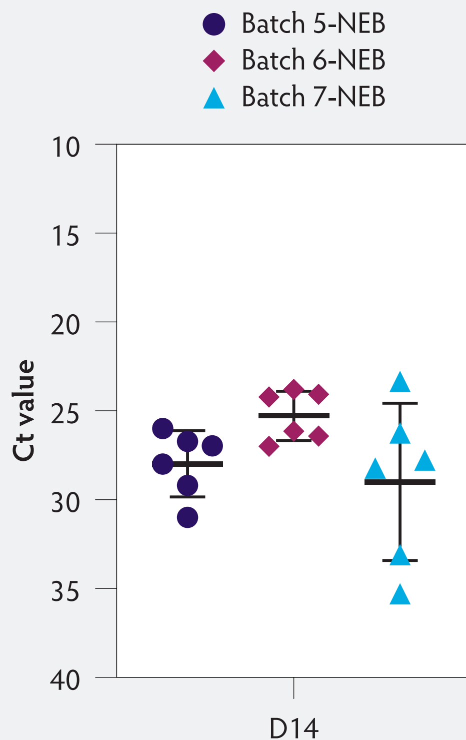


Figure 2: Tracheobronchial swab (TBS) sampling two weeks (D14) post exposure to *M hyopneumoniae* using nebulization inoculation. Individual and mean (SD) Ct values of positive TBS pooled samples using a qPCR test for *M hyopneumoniae*. Ct = cycle threshold; qPCR = real-time polymerase chain reaction; NEB = nebulization.



the pathogen. To control the time of infection with *M hyopneumoniae* and minimize the likelihood of bacterial shedding at the first farrowing, early controlled exposure has been attempted by administering lung tissue homogenate containing *M hyopneumoniae* to replacement gilts.[20,21,24,27] Inoculation with lung homogenate prepared from infected pigs might have the potential to introduce adventitious agents and aggravate the inflammatory response due to the administration of foreign antigens.[31] While the use of *M hyopneumoniae* pure culture would avoid these concerns, this bacterium is notoriously fastidious, and bacterial culture remains challenging and time consuming.[32] Moreover, potential legal aspects could arise from the use of bacterial isolates at farm level.

To intentionally infect pigs with *M hyopneumoniae*, the IT inoculation route has been extensively used under experimental settings.[26] The IT route is expected to apply a greater inoculum volume to the pig's lower respiratory tract, achieving greater infectious doses in shorter times and promoting an earlier *M hyopneumoniae* colonization.[33] Nevertheless, IT application is labor intensive, time consuming, and invasive. Practically, these factors present a great challenge to implementation of this method on a large scale in the swine industry. Another option would be the controlled exposure of naïve gilts to knowingly shedding animals (seeders). Roos et al[23] concluded that 6 seeders infected via IT were required in a group of 10 gilts for successful exposure to *M hyopneumoniae* in a 4-week exposure period. Due to the high ratio of infected animals needed and the unfeasibility of the IT methodology under field conditions, practical alternatives to ensure infection with *M hyopneumoniae* are needed. While NEB is supposed to closely mimic the natural conditions of *M hyopneumoniae* infection, it may also pose some constraints such as biosecurity and biocontainment issues, or lower dosage accuracy. In earlier work, pigs inoculated via IT displayed a significantly earlier upper respiratory tract colonization of *M hyopneumoniae* compared to those inoculated through NEB using individual exposure via an inhalation mask.[33] This finding could suggest a certain time lag in *M hyopneumoniae* infection dynamics partly due to the method of pathogen exposure. The present study assessed the validity of NEB to purposely expose naïve gilts to *M hyopneumoniae* in a field context.

In this framework, efficacy of NEB to infect gilts was evaluated by collecting TBS after exposure (D14 and D28) and testing pooled TBS by qPCR.

Tracheobronchial swab sampling in combination with qPCR testing for *M hyopneumoniae* has been introduced as an innovative technique to consistently detect the pathogen in different contexts.[28,34-38] It has been suggested that TBS is the most sensitive antemortem sample available to assess *M hyopneumoniae* prevalence in a pig population.[35,38] In our study, 30 TBS were collected per batch and time point. This sample size is widely used in the field to detect at least 1 positive from a population of 1000 pigs, assuming a 10% prevalence and 95% CI.[39] Applying this to the present research, at least one negative exposed pig per batch would have been detected by using this sample size. However, to significantly reduce the number of tests and associated costs, samples were submitted for testing in pools of 5. While this dilution step has been proposed as the preferential approach for field studies collecting TBS,[24,35,40] pooling more than one animal for each sample minimizes the effects of biological variation between individuals. In this context where high prevalence and low Ct values are expected, there is a risk of not detecting negative animals in a pool. This can certainly be understood as a limitation of the study as it can give false confidence about the data significance. Nevertheless, there is evidence in the literature that prove it unlikely that the pooled positive samples from this study were mainly composed of negative individual TBS. According to Sponheim et al,[38] the cumulative incidence of *M hyopneumoniae* infection, as detected by individual TBS, was 100% at about 14 weeks post infection in pigs inoculated via IT. A previous work using a mechanical fogger to expose gilts to *M hyopneumoniae* reported 100% positive individual TBS between 8 and 11weeks post fogging.[25] More recently, 100% of pooled TBS were PCR positive to *M hyopneumoniae* at 14 days post infection in pigs also exposed by NEB.[24] In this latter case, all samples were tested individually and every sample was confirmed PCR positive.

Overall, data obtained in the present study support the idea that NEB may be a convenient and effective methodology to infect gilts with *M hyopneumoniae* for acclimation purposes. Tracheobronchial swab pooled samples revealed no statistically significant differences in proportion of positives or in mean bacterial loads between batches early after exposure via IT and NEB. Although real prevalence of negative animals to *M hyopneumoniae* after exposure could not be addressed, the only pooled TBS samples that tested negative were collected on D28 from gilts exposed by both the IT and NEB routes. Due to the high analytical sensitivity yield by qPCR, all individuals within the negative pools are generally considered negative. However, and using PRRSV as an example, about 6% of the samples that would be detected by reverse-transcriptase PCR on individual serum would be missed if they were run in pools of 5.[41] In processing fluids, samples with initial Ct values of 35 would fall above the suspect threshold if further diluted.[42] However, pools that test positive indicate that at least one individual within each pool is positive, and individual retesting of each specimen is needed to discern between positives and negatives. Unfortunately, retesting of individual TBS samples could not be performed in this study as prepooled samples were not available. Moreover, to the knowledge of the authors, there is no literature assessing the changes in Ct values of *M hyopneumoniae* positive TBS pools due to the presence of negative samples. In consequence, the existence of negative subpopulations after exposure to *M hyopneumoniae* by the NEB technology cannot be discarded. Whether such negative subpopulations shortly after exposure influence the efficacy of acclimation strategies in reducing *M hyopneumoniae* shedding and prevalence of disease in downstream flow is unexplored and needs to be addressed in future work. Another scenario would be a herd undergoing an *M hyopneumoniae* elimination protocol. In this case, the presence of susceptible subpopulations represents a major risk for program failure,[43] which emphasizes the need to develop accurate diagnostic protocols to determine the success of *M hyopneumoniae* exposure. In summary, tailored diagnostic protocols are needed to reach the objective pursued with each acclimation strategy, which can be either control (low prevalence) or eradication of the infection.

Besides the inoculation methodology, successful exposure to *M hyopneumoniae* was also observed irrespective of the inoculum bacterial concentration. Thus, different titrations (expressed as genome copies per mL) of the final lung tissue homogenates were obtained, but no significant differences in proportion of positivity or in bacterial loads from TBS pooled samples were detected between any of the batches. While many other factors are probably involved, qPCR is not indicative of the bacterium viability in the inoculum as DNA fragments have been reported to be present in culture for long periods even when *M hyopneumoniae* cells are no longer viable.[44]

Mycoplasma hyopneumoniae-infected pigs via IT can potentially excrete the bacterium for up to 214 days following initial infection,[14] though the duration of shedding could vary in naturally infected gilts under field conditions.[29] In the present study, no excretion data was obtained late post exposure. This information for NEB also is lacking in the literature, thus, whether the excretion pattern is different in pigs exposed to *M hyopneumoniae* by NEB remains unknown and should be the subject of further investigation. The age of exposure has major importance when the goal is to obtain nonshedding gilts by the time of first farrow. Gilts from the present study entered and left the GDU at approximately 7 and 28 weeks of age, respectively, and the age at first mating was about 35 weeks. Considering a shedding duration of 214 days (approximately 31 weeks) and that the acclimation process started around 10 to 13 weeks of age, the protocol used in this study would likely have ensured the elimination of *M hyopneumoniae* shedding at first farrowing, as suggested by Pieters and Fano.[15] Regrettably, *M hyopneumoniae* status of gilts at first farrowing was not checked, therefore, it remains unknown whether this acclimation protocol was effective in reducing bacterial shedding at that critical time. Also, there are numerous factors that could impact the duration of *M hyopneumoniae* shedding, for instance, the immunological status of the infected animals. The gilts enrolled in this study were vaccinated against *M hyopneumoniae* before their entrance to the GDU. In naturally infected gilts under field conditions, a lower duration of *M hyopneumoniae* shedding has been suggested in vaccinated gilts when compared to their nonvaccinated counterparts.[29] The latter, however, should be corroborated in experimentally inoculated animals where the exact time of exposure to *M hyopneumoniae* is known. Still, protocols including exposure of vaccinated gilts could be advantageous for reducing acclimation timings.

Implications

- Gilt acclimation to *M hyopneumoniae* is key for sustainable EP and PRDC control.
- Controlled exposure to *M hyopneumoniae* may be a complementary acclimation method.
- Nebulization could be used consistently to expose gilts to *M hyopneumoniae*.

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Conflict of interest

None reported.

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* Non-refereed references

Conversion tables

Weights and measures conversions

Common (US)	Metric	To convert	Multiply by
1 oz	28.35 g	oz to g	28.4
1 lb (16 oz)	453.59 g	lb to kg	0.45
2.2 lb	1 kg	kg to lb	2.2
1 in	2.54 cm	in to cm	2.54
0.39 in	1 cm	cm to in	0.39
1 ft (12 in)	0.31 m	ft to m	0.3
3.28 ft	1 m	m to ft	3.28
1 mi	1.6 km	mi to km	1.6
0.62 mi	1 km	km to mi	0.62
1 in^2	6.45 cm^2	in^2 to cm^2	6.45
0.16 in^2	1 cm^2	cm^2 to in^2	0.16
1 ft^2	0.09 m^2	ft^2 to m^2	0.09
10.76 ft^2	1 m^2	m^2 to ft^2	10.8
1 ft^3	0.03 m^3	ft^3 to m^3	0.03
35.3 ft^3	1 m^3	m^3 to ft^3	35
1 gal (128 fl oz)	3.8 L	gal to L	3.8
0.264 gal	1 L	L to gal	0.26
1 qt (32 fl oz)	946.36 mL	qt to L	0.95
33.815 fl oz	1 L	L to qt	1.1

Temperature equivalents (approx)

°F	°C
32	0
50	10
60	15.5
61	16
65	18.3
70	21.1
75	23.8
80	26.6
82	28
85	29.4
90	32.2
102	38.8
103	39.4
104	40.0
105	40.5
106	41.1
212	100

°F = (°C × 9/5) + 32
°C = (°F - 32) × 5/9

Conversion chart, kg to lb (approx)

Pig size	Lb	Kg
Birth	3.3-4.4	1.5-2.0
Weaning	7.7	3.5
	11	5
	22	10
Nursery	33	15
	44	20
	55	25
	66	30
Grower	99	45
	110	50
	132	60
Finisher	198	90
	220	100
	231	105
	242	110
	253	115
Sow	300	135
	661	300
Boar	794	360
	800	363

1 tonne = 1000 kg
1 ppm = 0.0001% = 1 mg/kg = 1 g/tonne
1 ppm = 1 mg/L