RESEARCHREPORT
SWINE HEALTH

Title: *Achieving safe introduction of naïve gilts into M. hyopneumoniae endemically infected herds: Protecting existing sows, exposing incoming gilts, and minimizing shedding to the offspring* - **NPB # 17-024** Revised

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Industry summary

Objective 1
Gilt acclimation for *M. hyopneumoniae* has been proposed as a strategy to control this respiratory bacterium in breeding herds. Despite the fact that vaccination is currently the most utilized strategy for gilt acclimation, intentional exposure to *M. hyopneumoniae* is increasing in popularity in some parts of the world. The objective of this study was to compare the effectiveness and consistency of four *M. hyopneumoniae* exposure methods based on *M. hyopneumoniae* detection, disease development, and humoral immune response against the bacterium. Forty *M. hyopneumoniae* negative gilts were distributed into five experimental groups according to the inoculation methods: 1) Intra-tracheal catheter (ITc; n=5); 2) Laryngo-Tracheal Mucosal Atomization device (LTMA; n=10); 3) Intranasal device (INd; n=10); 4) Rope chewing (RCh; n=10); 5) Mock-inoculated (MI; n=5). Laryngeal swabs and blood samples were collected at 0, 14, and 28 days post-inoculation (dpi) to detect *M. hyopneumoniae* by rt-PCR and to assess the presence of antibodies by ELISA. All gilts were euthanized at 28 dpi for lung lesion assessment and sample collection. Gilts in MI group remained negative throughout the study. A statistical difference in detection of *M. hyopneumoniae* was observed between ITc and LTMA compared to INd and RCh at 28 dpi. All gilts remained seronegative at 28 dpi, except two in ITc (2/5, 40%) and five in LTMA (5/10, 50%). The LTMA and ITc groups showed numerically higher cough indices than INd and RCh. Under the conditions of this study, LTMA route was equally effective in inducing *M. hyopneumoniae* infection as ITc (standard infection model). In conclusion, the LTMA for *M. hyopneumoniae* infection could represent a novel, safe and effective route, offering ease of application to inoculate gilts against this bacterium.

Objective 2
Results indicate that *M. hyopneumoniae* vaccination induced a rapid adaptive cellular immune response as early as two weeks post-vaccination, whereas humoral responses were only detected four weeks post-vaccination . Furthermore, they revealed an independent dynamic of specific T-cell and antibody responses to *M. hyopneumoniae* vaccination and infection. Nonetheless, no significant differences in cellular immune responses between different treatment groups, post-infection was observed suggesting that timing and non-specific PMBC stimulation might have impacted our results. Although, *M. hyopneumoniae* induced cell-mediated immunity has been associated with protection from disease, limited evidence is available for cell-mediated immunity to *M. hyopneumoniae* after vaccination. Therefore, future experiments on antigen specific T cell and B cell responses will help better understand cellular immune function in hosts and will reveal alternative cellular assay based methods to measure vaccine efficacy.

Keywords: Gilt, acclimation, *Mycoplasma hyopneumoniae,* replacement gilt, exposure

Introduction:
Mycoplasma hyopneumoniae (M. hyopneumoniae) is the causative agent of enzootic pneumonia, a respiratory condition prevalent in a high proportion of swine herds worldwide, including the US (reviewed by Maes et al., 2008; Thacker and Minion, 2012). Most commercial herds are endemically infected with M. hyopneumoniae and may not consider elimination of the pathogen as a viable option within their production systems. In those cases, which include a significant number of swine herds, appropriate management practices conducing to maintaining an endemic infection of *M. hyopneumoniae* are strictly necessary in order to keep uniformly exposed groups of pigs. In other words, keeping a stable reproductive herd is central to maintain the overall herd status. On the other hand, producers and veterinarians have quantified the economic impact of disease free production and the economic benefits of growing M. hyopneumoniae free pigs have been realized (Yeske 2015; Schwartz 2015). For those reasons *M. hyopneumoniae* elimination efforts have become a priority in the industry (Holst et al., 2015).
Sow-to-piglet transmission of *M. hyopneumoniae* constitutes a crucial epidemiological event in the life of the pig (Calsamiglia and Pijoan, 2000; Sibila et al., 2009). Piglets are born free of M. hyopneumoniae and may become exposed during the lactation period (Calsamiglia and Pijoan, 2000), at which end a variable proportion of piglets will be colonized (Fano et al., 2005; Villarreal et al., 2009). It has been previously demonstrated that the level of piglet colonization at weaning age in a given group is correlated with the severity of clinical disease at market age (Fano et al., 2007). Therefore, control measures directed to decrease M. hyopneumoniae prevalence at weaning seem to be necessary to

potentially diminish problems associated with the clinical presentation. Recently, several studies have focused on the identification of factors that affect the level of *M. hyopneumoniae* colonization at the end of the lactation period, at the environmental, herd and individual level. Results from several investigations suggest a strong influence of maternal shedding of M. hyopneumoniae during the lactation period in the colonization at weaning age with this pathogen (Nathues et al., 2013; Pieters et al., 2014).

Gilt and sow shedding of *M. hyopneumoniae* during lactation may be the result of several events occurred earlier in the life of the dam and/or the group of dams, which may include age at exposure and infection with *M. hyopneumoniae*, immune status, previous medications, herd biosecurity, and perhaps more importantly, acclimation processes and management practices used for introduction of negative replacement animals into endemically infected herds (Dalquist 2014).

In general, the purpose of acclimation processes is to manage the gilts in a way that they achieve a health status similar to that of the recipient herd, and therefore do not pose a risk to themselves, to the already established herd, or to the progeny of those newly introduced animals (Pieters and Fano, submitted). Management protocols have been developed for gilt acclimation to swine pathogens like PRRSV for example, which have proven to be effective to keep sow herd stability. However, management strategies for proper gilt acclimation to M. hyopneumoniae do not appear to be highly effective or well understood, thus sow herd destabilization occurs fairly frequently, which often translates into variable colonization at weaning age and severe clinical presentation in the finishing phase.

We believe that the establishment of herd immunity against *M. hyopneumoniae* in infected breeding herds will decrease the *M. hyopneumoniae* prevalence in the sows, which will decrease the likelihood of *M. hyopneumoniae* transmission to pre-weaning piglets. For the purpose of this investigation, we assume that the higher the number of sows/gilts immunized against *M. hyopneumoniae* the lower the pressure of infection of M. hyopneumoniae in the breeding herd and in pre-weaning piglet's populations. Considering that *M. hyopneumoniae* bacterins do not induce protective immunity against M. hyopneumoniae (Haesebrouck et al., 2004) there is a need to seek for alternative methods (i.e. exposing pigs with live *M. hyopneumoniae*) to induce protective immunity against subsequent *M. hyopneumoniae* infections.

In summary, assuring a stable sow herd as it relates to *M. hyopneumoniae* infection seems vital for effective control of downstream respiratory disease in endemic production systems. In addition, elimination programs for M. hyopneumoniae rely on an affective exposure to the pathogen at a given time point in order to mark the "zero" that is used to start herd closures for eradication. Thus, developing a practical protocol for safe and effective exposure to *M. hyopneumoniae* of incoming gilts is of paramount importance for disease control. To the best of our knowledge, there are no described strategies for effective gilt exposure to *M. hyopneumoniae* under field conditions. Therefore, the overall goal of this investigation is to develop a protocol for consistent, safe, and

effective acclimation of M. hyopneumoniae negative gilts that can be used at the farm level.

Objectives: A series of studies, addressing specific objectives will contribute to the overall goal of developing a protocol for exposing, detecting and retaining *M. hyopneumoniae* infected gilts, while protecting incoming gilts and existing sows, and minimizing bacterial shedding to the piglets. The specific objectives are:

1) To propose a protocol for timing of gilt introduction (completed; 50-350 gilt acclimation)
2) To determine the optimum seeder-to-naïve gilt ratio for successful exposure of naïve gilts to *M. hyopneumoniae* during a 4-week exposure period (completed; a 1:1 ratio for successful exposure)
3) To evaluate the effect of gilt flow (continuous flow vs. AI/AO) on the gilt exposure and recovery from *M. hyopneumoniae* prior to their first farrowing (funded)
4) **To compare methods for safe and practical gilt exposure to *M. hyopneumoniae*** (in this proposal)
5) To investigate the effect of previous infection with a *M. hyopneumoniae* strain in protection against a subsequent infection with a different strain (incomplete; ongoing investigation)
6) **To determine the accuracy of a diagnostic protocol for detecting exposure to live *M. hyopneumoniae* in previously vaccinated animals** (in this proposal)
7) To evaluate the feasibility and economic impact of a Reverse Test and Removal (RTR) protocol for gilt selection prior to introduction in the reproductive herd (to be proposed for year 2)

The combination of results obtained from the different studies, addressing each specific objective, will be used for the development of a protocol for safe gilt introduction into endemically infected herds.

Objective 4

Methods

Animal selection and housing

Forty 5-week old gilts were randomly selected from an experimental herd that has been free of *M. hyopneumoniae* for many years based on serological testing. Selected gilts were individually ear-tagged and confirmed ELISA (IDEXX) negative to *M. hyopneumoniae* at -13 day post inoculation (dpi). At -4dpi, gilts were transported to the BSL-2 isolation facilities at the University of Minnesota, St. Paul Campus. The study was performed after approval by the Institutional Animal Care and Use Committee of the University of Minnesota. Lung homogenate (strain 232) was obtained from Iowa State University.

Experimental design

At -4 dpi, gilts were randomly distributed, blocked by litter, into five groups (one group per room) according to following inoculation route: 1) Intra-tracheal catheter (ITc; n=5); 2) Laryngo-Tracheal Mucosal Atomization (LTMA; n=10); 3) Intranasal with device (INd; n=10); 4) Rope chewing (RCh; n=10); 5) Mock-inoculated (MI; n=5). Gilts in groups 1-4 were inoculated at 0 dpi with 10 mL of *M. hyopneumoniae* lung homogenate containing strain 232 (Minion et al., 2004) at a concentration of $1x10^5$ CCU/mL, whereas MI group

was inoculated with 10ml of Friis media (Kobish and Friis, 1996). At the end of the study (28 dpi) all gilts were humanly euthanized with a barbiturate overdose.

Experimental inoculation procedures

ITc inoculation was performed on snared gilts using a Dover™ PVC Urethral Catheter (Covidien, Minneapolis, MN, USA) for inoculum delivery into the trachea, and a laryngoscope and a mouth gag for visualization (Pieters et al. 2009). For the LTMA inoculation route, a MADgic Laryngo-Tracheal Mucosal Atomization Device (Teleflex Medical Europe Ltd, Westmeath, Ireland) was attached to a 20 mL syringe containing the lung homogenate. The lung homogenate (10 mL) was delivered to the laryngeal area of snared gilts by depressing the syringe, forcing the lung homogenate through the atomizer to the larynx. For the INd inoculation, a MAD Nasal Intranasal Mucosal Atomization Device (Teleflex Medical Europe Ltd., Westmeath, Ireland) was attached to a 20 mL syringe containing the lung homogenate (10 mL). The gilts were snared and the lung homogenate was delivered intranasally by depressing the syringe and forcing the homogenate through the atomizer. The atomizing device is currently used in human medicine to apply anesthetics to the larynx of patients prior to intubation. The RCh group was inoculated with a homemade device utilizing a 4 lb. Double-Tuf salt block Feeder (Miller Manufacturing Company, Glencoe, MN, USA), cotton ropes, and metal washers. Three of the ropes were cut to hang 15 cm below the bottom of the feeder and three were cut to hang 7.5 cm below the bottom of the feeder. The longer ropes were hung along the back of the feeder and the shorter ropes were hung along the front of the feeder. As gilts chewed on the longer ropes, the shorter ropes reached their noses. Three of these devices were built and hung throughout the pen so that each gilt had adequate access to chew on a device. The ropes on the devices were dipped in the lung homogenate and the gilts were immediately allowed to chew on the ropes. After two minutes, the ropes were again dipped with the homogenate and the pigs again allowed to chew. This process was repeated until the 100 mL of homogenate (10 ml per gilt) was gone. Each gilt was observed to chew at least once on the ropes. The MI group was inoculated using an identical method to the ITc group, substituting the Friis media for the lung homogenate. No anesthesia was required for any inoculation procedure.

Clinical examination

Gilts were monitored for respiratory clinical signs (dry cough) for 15 minutes daily from 10 until 25 dpi. This monitoring was performed daily and a coughing index (CI) was calculated as previously described Nathues et al. (2012).

Sample collection, processing and testing

Laryngeal swabs (LS) and blood samples (BS) were collected 0, 14 and 28 dpi to detect *M. hyopneumoniae* by rt-PCR and to assess *M. hyopneumoniae* antibodies by ELISA, respectively. Laryngeal swabs were collected using sterile swabs (BBL™ CultureSwab™, Sparks, MD, USA) as previously described by Pieters et al. (2017) and frozen at -20°C until processed. These were processed for DNA extraction with MagMAX™-96 Viral RNA isolation kit and MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA) and tested by rt-PCR with VetMAX™ qPCR Master mix and VetMAX™ *M. hyopneumoniae* reagents kit (Life Technologies, Grand Island, NY, USA), following manufacturer's protocol. All samples were run in duplicate. Samples were considered positive for real time PCR when Ct was lower than 37, suspect if Ct values were 37 to 39, and positive if greater than 39. Samples initially considered suspect were re-tested and classified based on the second result.

Blood was collected from jugular vein using BD Serum Vacutainers® (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Once in the laboratory, blood samples were

centrifuged at 1500g x 10 min at 4°C for serum separation. Serum was aliquoted, stored at -20°C and later tested with an ELISA test (IDEXX Laboratories, Westbrook, ME, USA) for detection of antibodies against *M. hyopneumoniae*, following manufacturer's protocol. Samples were considered positive when S/P ratio ≥ 0.4.

At necropsy (28 dpi), lungs were scored on the percentage of affected tissue associated with *M. hyopneumoniae* infection as described by Pointon et al. (2012). Bronchial swabs (BS) were collected to be tested with rt-PCR as previously described for LS. Three lung samples were collected from gilts and were fixed in 10% formalin for histopathological studies utilizing the method described by Morris *et al.* (1995) and Calsamiglia *et al.* (2000). Lung tissues from one gilt in the INd group and one gilt in the LTMA group were not found on histopathological review and are not included in the histopathologic results.

Statistical analysis

Bivariate analysis using the Kruskal-Wallis test was applied for median comparison of ELISA results among groups at different sampling points. The Chi square test were applied to evaluate rt-PCR results, lung lesions and coughing index between different groups at different sampling points. When significant results were obtained, *a posteriori* contrast analysis 2 to 2 was performed. Statistical analysis was performed using PSPP v.18 (PSPP, Chicago, USA). The significance level was set to $p<0.05$.

Results

Clinical examination and coughing index

The ITc group showed the highest coughing index over the observation period, followed by LTMA, INd and RCh groups. In contrast, the negative control (MI) group did not cough at all (Table 1).

Table 1. Number of total coughs, coughing indices and standard deviation in different studied groups during 15 min of observation from 10 to 25 days post inoculation.

Group	Total Coughs	Coughing Index	Standard Deviation
ITc	51	0.043	0.025
LTMA	91	0.038	0.013
INd	21	0.009	0.008
RCh	8	0.003	0.006
MI	0	0	0

Detection of M. hyopneumoniae by rt-PCR in laryngeal and bronchial swabs

At 0 dpi, all gilts were rt-PCR negative on LS. The RCh and MI groups remained negative throughout the study (Table 2). On 14 dpi, 1 out of 5 (20%) from ITC, 2 out of 10 (20%) from LTMA and 1 out of 10 (10%) from INd groups were *M. hyopneumoniae* positive in LS. On day 28 dpi, 3 out of 5 (60%) gilts in ITc, all (10/10, 100%) from LTMA and 4 out of 10 from INd (40%) were LS positive to *M. hyopneumoniae.* Finally, all BS collected at 28 dpi from ITc (5/5, 100%) and LTMA (10/10, 100%) were *M. hyopneumoniae* positive, whereas 4 out of 10 (40%) from INd group resulted positive. Significant differences in ITc and LTMA compared to INd, ChR and MI groups were detected in LS and BS at 28 dpi.

Table 2. Detection of *M. hyopneumoniae* by rt-PCR (Ct mean±SD) in laryngeal swabs taken from studied gilts over time.

	Sampling points							
	LS						BS	
Inoculation method	0 dpi		14 dpi		28 dpi		28 dpi	
	Prop (%)	Ct	Prop (%)	Ct	Prop (%)	Ct	Prop (%)	Ct
ITc	0/5 (0.0)[a]	NA	1/5 (20.0)[a]	38.8±1.3	3/5 (60.0)[a]	34.6±3.4	5/5 (100.0)[a]	29.4±2.5
LTMA	0/10 (0.0)[a]	NA	2/10 (20.0)[a]	38.7±0.6	10/10 (100.0)[a]	34.6±3.3	10/10 (100.0)[a]	27.8±2.8
INd	0/10 (0.0)[a]	NA	1/10 (10.0)[a]	35.7±0.0	4/10 (40.0)[b]	34.9±2.9	4/10 (40.0)[b]	29.6±1.7
RCh	0/10 (0.0)[a]	NA	0/10 (0.0)[a]	NA	0/10 (0.0)[ab]	NA	0/10 (0.0)[b]	NA
MI	0/5 (0.0)[a]	NA	0/5 (0.0)[a]	NA	0/5 (0.0[b]	NA	0/5 (0.0)[b]	NA

Detection of antibodies against M. hyopneumoniae in serum
All studied gilts remained seronegative over the study with the exception of gilts from the ITc (2/5, 40%) and LTMA (5/10, 50%) groups which seroconverted at 28 dpi.

Macroscopic and microscopic lung lesions
M. hyopneumoniae-like lung lesions were observed among in all inoculated groups as well as in one gilt from MI group. Percentage of gilts showing lung lesions associated with *M. hyopneumoniae* and range of affected surface area is detailed in Table 3. Regarding the microscopic lesions, the histopathological scores per group are also shown in Table 3.

Table 3. Proportion (%) of gilts showing macroscopic lung lesions associated with *M. hyopneumoniae*, range of affected surface area and microscopic scores at 28 dpi.

Inoculated group	Macroscopic			Microscopic				
	Proportion (%) of gilts showing lung lesions	Median of affected lung surface area (%)	Range of affected lung area	Percentage of gilts showing different lung lesion scores[a]				
				0	1	2	3	4
ITc	5/5 (100%)	11	1-24	0	20	80	0	0
LTMA	10/10 (100%)	8	4-26	0	22	67	11	0
INd	5/10 (50%)	1	1-21	56	44	0	0	0
RCh	3/10 (3%)	2	1-11	40	60	0	0	0
MI	0/5 (0%)	0	-	0	0	0	0	0

[a]Lung lesions were scored from 0 to 4 according to previously described criteria (Morris et al., 1995; Calsamiglia et al. 2000)

Interpretation/discussion
Gilt acclimation strategies focused on reducing the *M. hyopneumoniae* shedding between the dams and their offspring at farrowing has been proposed as one of the most important points to control EP within farms (Pieters and Fano, 2016). Ideally, a proper gilt acclimation is described as a process in which all gilts get infected and subsequently have time to recover from the disease and shedding during acclimation (Pieters and Fano, 2016). Due to the long persistence (Pieters et al., 2009) and the low transmission

rate of *M. hyopneumoniae* (Meyns et al., 2004), getting gilts infected early enough to recover before the end of acclimation under natural conditions represents an important challenge (Roos et al., 2016). Currently, in the US, intentional gilt exposure to *M. hyopneumoniae* is commonly used to facilitate the infection process (Fano and Payne, 2015; Sponheim et al., 2017; Garza-Moreno et al., 2018). However, no information about the efficacy, consistency or safety of the methods used for gilt exposure is available. Therefore, the objective of the present study was to assess different inoculation methods for achieving *M. hyopneumoniae* infection in gilts.

The usage of ITc and INd models to induce EP under experimental conditions has been described by previous studies. Nevertheless, to the author's knowledge, this is the first time that LTMA and RCh methods are assessed for causing *M. hyopneumoniae* infection. Clinical examination of inoculated gilts measured by coughing indexes indicated a more severe disease in ITc and LTMA groups compared to INd and RCh. Considering that *M. hyopneumoniae* causes a slowly progressing disease, and coughing scores were only collected up to 25 dpi, all colonized gilts may not have had enough time to show clinical signs. On the other hand, the lack of coughing in the MI group supports the idea that the biosecurity practices in place succeeded in preventing transmission of *M. hyopneumoniae* between study rooms.

M. hyopneumoniae detection in LS by rt-PCR showed that the highest proportion of colonized gilts was found in the LTMA group at 28 dpi, followed by ITc at the same sampling point. This finding suggests that the usage of the LTMA device seems to be more effective for *M. hyopneumoniae* gilt infection and could lead to more effective gilt acclimation. Similarly, results of *M. hyopneumoniae* detection in BS at 28 dpi showed all gilts inoculated using LTMA and ITc were positive. These findings are in agreement with previous studies that concluded laryngeal sampling is a sensitive and reliable method for *M. hyopneumoniae* detection in live pigs, but *post mortem* bronchial swabs are the most sensitive method (Pieters et al., 2017). A potential hypothesis for the increase of *M. hyopneumoniae* colonization over the ITc route which delivers the inoculum very close to the same area may be the difference in particle size of the inoculum. The LTMA device produces 30-100 µm particles while the ITc catheter delivers a stream of homogenate. These smaller particles may result in inoculum reaching areas of the respiratory tract that the stream of fluid from the catheter might not reach.

On the other hand, the low proportion of rt-PCR *M. hyopneumoniae* positive gilts in the INd group suggests that intranasal inoculation is an ineffective route of inoculation. Although the intranasal device also produces 30-100 µm particles, they are delivered to a more rostral location in the respiratory tract than the ITc and LTMA device. Moreover, intranasal inoculation resulted in more wastage of the inoculum as gilts immediately sneeze upon inoculation and fluid could be visualized leaving the nostrils. This fact indicated that a portion of the original inoculum volume was immediately removed from the respiratory tract and thus, it might cause low colonization rates. Similarly, rt-PCR results in the RCh group indicated that hanging ropes soaked in lung homogenate was also an ineffective route to induce EP in gilts. Although this is a very easy method of presenting gilts with inoculum, it does not produce the desired results. Finally, it is important to note that MI group remained *M. hyopneumoniae* negative by rt-PCR throughout the study. This finding confirmed biosecurity procedures were effective in preventing the transmission of *M. hyopneumoniae* between different groups.

Detection of antibodies against *M. hyopneumoniae* is the most commonly used method to provide evidence of exposure to the pathogen. Nevertheless, given that the time required for pigs to seroconvert is variable, seronegative animals may not be *M.*

hyopneumoniae negative in early infections (Maes et al., 2017; Garza-Moreno et al., 2018). Indeed, in agreement with these previous results, the positive proportion of gilts detected in the ITc and LTMA groups on LS and BS at 28 dpi was not equivalent to the seropositive gilt proportion by ELISA. In addition, ELISA test did not detect any of the rt-PCR positive gilts in the INd group. These results show that assessing antibody levels within 28 days from *M. hyopneumoniae* exposure is very inaccurate, suggesting that antibody and pathogen detection should be combined for *M. hyopneumoniae* diagnosis.

Although all inoculated groups developed lung lesions, the LTMA and ITc groups showed the highest mean gross lung lesion scores. This finding aligns with the rt-PCR, ELISA, and cough index results, supporting the idea that these are the most effective routes of inoculation. Interestingly, four gilts from RCh group and one gilt from the MI group showed a lung lesions that covered 2% to 10% of the surface of the lung. Nevertheless, it does not imply that these gilts were colonized with *M. hyopneumoniae* as cranio-ventral pulmonary consolidation (CVPC) is suggestive, but not exclusive of *M. hyopneumoniae* infection. Indeed, others pathogens such as *Pasteurella multocida* and/or swine influenza virus could also cause CVPC (Thacker and Minion, 2012).Nevertheless, rt-PCR and ELISA results combined with the lack of coughing strongly confirmed the observed lesion was not caused by *M. hyopneumoniae.* Unfortunately, in this study, the potential infections of others respiratory pathogens were not assessed.

Regarding microscopic evaluation of affected lung tissues, only one gilt from LTMA group showed a compatible histopathologic lesion to *M. hyopneumoniae* (category 3). The other inoculated groups, with the exception of MI group, showed varying levels of inflammation or other lesions non-specific to *M. hyopneumoniae* (categories 0 to 2). Taking these macroscopic and histopathological findings together with rt-PCR, ELISA and clinical examination results, it was confirmed that the diagnosis based only on macroscopic lung lesions and histopathology could not detect *M. hyopneumoniae* early infections (Calsamiglia et al., 2000). Therefore, a combination of various techniques is needed.

In conclusion, obtained results suggest that ITc and LTMA are the most effective methods for *M. hyopneumoniae* gilt exposure. Although both methods requires snaring gilts, in the author's opinion the LTMA device reduces snaring time because its rigidity allows quicker passage beyond the epiglottis than ITc. This fact meant that the usage of the LTMA device was more user-friendly and practical to be used under field conditions. Therefore, the LTMA method seems to be an efficient inoculation route to induce MP in gilts, representing a potential exposure strategy during gilt acclimation for controlling *M. hyopneumoniae* in the field.

Objective 6

Experimental Design

Twenty 21-day old commercial gilts from *Mycoplasma hyopneumoniae* negative sow farm were selected and weaned into a nursery. A day after weaning (-28 days post inoculation, [dpi]) and -11 dpi , 10 gilts were vaccinated with a known commercial vaccine whereas the remaining10 remained unvaccinated. At 46 days of age (-4 dpi), gilts were transferred to an isolation facility and allocated to four different groups: 1) vaccinated-intratracheal inoculated (Vax, catheter; n=5), 2) non-vaccinated-intratracheal inoculated (Nonvax, catheter; n=5), 3) vaccinated- mock inoculated (Vax, mock; n=5), 4) non-vaccinated- mock inoculated (Nonvax, mock; n=5). All gilts, except the mock groups, were inoculated with 10 mL of *M. hyopneumoniae* lung homogenate (Iowa State University, LI44-2) at 0 dpi. The mock groups were inoculated with 10ml of modified Friis media. Blood samples were collected at days -28, -12, 0, 14 and 28 dpi and peripheral blood mononuclear cells (PBMCs) were isolated. T-cell activation was then evaluated by quantification of the *M. hyopneumoniae* -specific IFN-γ secreting cell frequencies in PBMC as described previously using ELISPOT assays. Additionally, serum samples from whole blood was extracted and a commercial ELISA was performed for detecting *M. hyopneumoniae* antibodies.

Methods

Porcine peripheral blood mononuclear cell (PBMC) isolation

Whole blood was collected in vacutainer venous blood collection tubes coated with ethylenediaminetetraacetic acid (EDTA; Becton, Dickinson and Company, New Jersey) and diluted 1:1 with sterile 1X phosphate buffered saline (PBS; pH = 7.4). The diluted blood was overlaid on lymphocyte separation medium (Corning, New York) and separated by centrifugation at 2000 rpm for 30 min at room temperature (20-25^{o}C) with slow deceleration. After separation of red blood cells, PBMCs were washed once with PBS and once with culture medium (Gibco® RPMI Media 1640, Life Technologies Inc., Carlsbad, CA) containing 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were stained with 0.4% Trypan blue (VWR International, Radnor, PA) and counted on a hemocytometer (Huang et al., 2014).

Interferon gamma (IFNγ) ELISPOT assay

MultiScreen filter plates (EMD Millipore Corp. Billerica, MA) were coated with 100 µL/well (1:60 dilution in PBS) anti-porcine IFNγ capture antibodies (R&D Systems, Minneapolis, MN; lyophilized powder dissolved in 1ml reagent diluent - 1% bovine serum albumin in PBS), incubated overnight at 4°C, washed six times with PBS and blocked with blocking buffer (1% BSA+ 5% sucrose in PBS) at room temperature for 2 h. After the blocking was done, the wells were washed six times with PBS and rinsed with culture medium (Gibco® RPMI Media 1640). Fifty microliters of PBMCs (10^7/ml) were dispensed into each well and incubated with 10 µg/mL whole cell sonicated *M. hyopneumoniae* crude antigen. Concanavalin A (ConA; 10 µg/mL) stimulated PBMCs were included as

positive controls whereas cells stimulated with the modified Friis Medium (*M. hyopneumoniae* growth medium: used for crude antigen preparation) were used as mock controls. Additionally, the unstimulated cells served as negative controls. The cells were incubated for 20 hours at 37°C in the presence of 5% CO_2. Each sample was assayed in triplicate. Cells were removed, and plates were washed twice with double distilled water and six times with wash buffer (PBS with 0.05% Tween 20), then incubated with biotinylated polyclonal IFNγ detection antibody (1:60 dilution in PBS; R&D Systems; lyophilized powder dissolved in 1ml reagent diluent) for 2 h at room temperature. Plates were washed six times with wash buffer and incubated with streptavidin conjugated to alkaline phosphatase (R&D Systems) in reagent diluent at room temperature for 2 h. Plates were washed four times with wash buffer and twice with distilled water. Spots were developed by adding BCIP/NBT (R&D Systems) to each well according to the manufacturer's instructions and incubated in the dark for 30 min at room temperature. The wells were then rinsed three times with distilled water and the number of spots in each well was counted using Cellular Technology Limited (CTL) Elispot Reader and processed using ImmunoSpot® software (Cellular Technology Limited (CTL), Cleveland, OH).

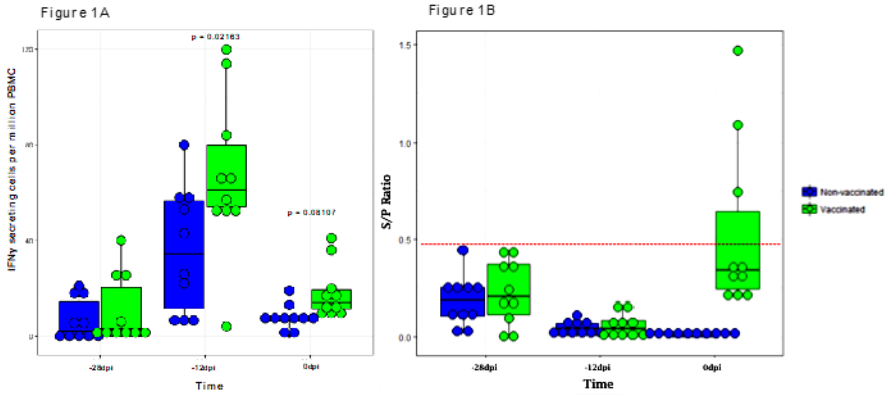
Number of *M. hyopneumoniae*-stimulated IFNγ secreting cells was calculated by subtracting the number of spots in unstimulated wells from those in *M. hyopneumoniae* stimulated wells. Likewise, the number of ConA- stimulated IFNγ secreting cells and mock stimulated cells were determined. Finally, the *M. hyopneumoniae*-specific responses were determined by subtracting the number of spots in mock stimulated wells from those in *M. hyopneumoniae* stimulated wells. The final number of spots was expressed as IFNγ secreting cells per million PBMC. (Huang et al., 2014) .

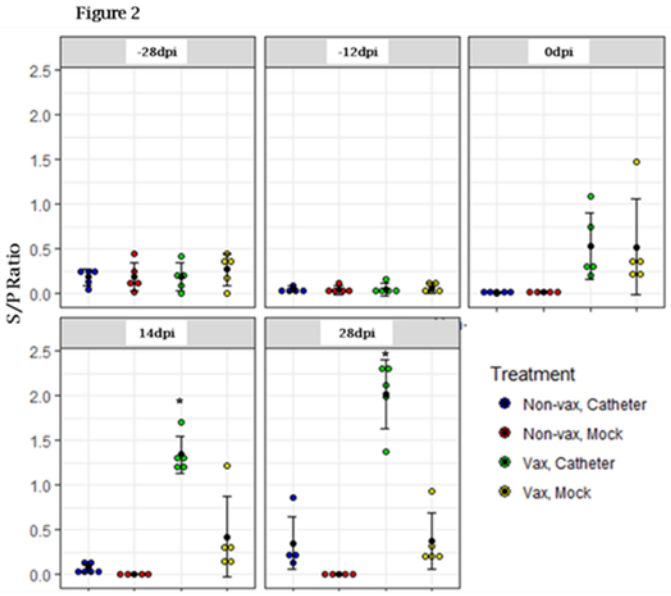
Antibody immune responses

Serum samples were tested for *M. hyopneumoniae* antibodies employing Idexx ELISA kits (IDEXX Laboratories, Westbrook, Maine, USA) following manufacturer's instructions. Positive and negative *M. hyopneumoniae* serological status were determined based on optical density (OD) of the sample to the positive ratio (S: P); S: P = (sample OD - negative control OD)/ (positive control OD - negative control OD). All samples were run in duplicate and sample means were used to determine the final S: P ratio. S: P ratios ≥0.4 was classified as positive; S: P ratios <0.4 were identified as negative (Pieters et al., 2017).

Results

The vaccination triggered an early and intense IFNγ secreting cell response as early two weeks post- first vaccination by (-12dpi) inducing the activation of peripheral lymphocytes (Figure 1A). However, the humoral immune response against *M. hyopneumoniae* remained undetectable until 4 weeks post- first vaccination (0dpi; Figure 1B and 2). On 0dpi, 7/10 among the vaccinated gilts remained negative (S/P values <0.4) in the ELISA assay (Figure 1B).





Furthermore, all the vaccinated gilts were seroconverted faster compared to unvaccinated animals on exposure to the pathogen which was evidenced with higher antibody titers at two weeks post-infection (14dpi, P<0.05; Figure 2).

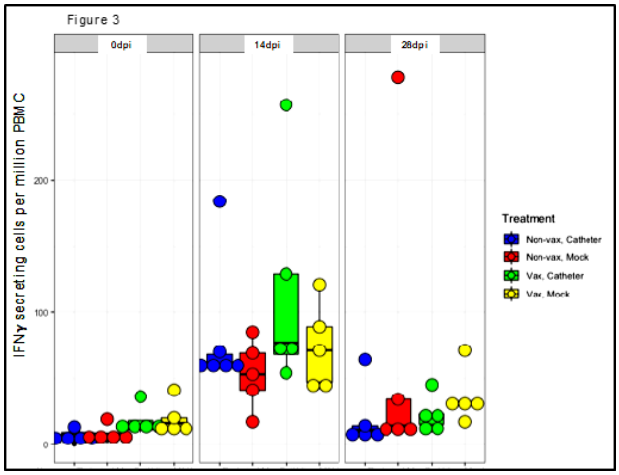
On the other hand, irrespective of vaccination status, there was no significant difference in IFNγ secreting cell response to pathogen or mock-medium challenge in gilts (Figure 3). This indicates that even when the gilts were inoculated with mock-medium, there were detectable non-specific cellular immune responses resulting in the activation of peripheral lymphocytes and IFNγ secretion.

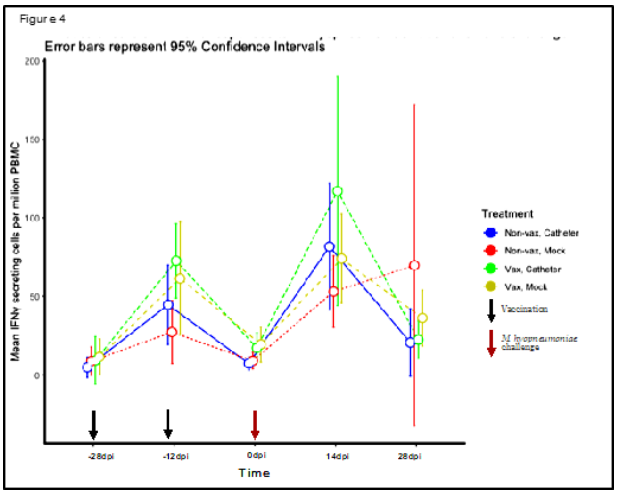
Additionally, the kinetics of IFNγ secreting PBMC proliferation after *M. hyopneumoniae* vaccination and infection was determined *in vivo*. The mean of the individual responses from all five gilts from different treatment groups were plotted in figure 4. Mean number of *M. hyopneumoniae* IFNγ secreting PBMCs were higher in vaccinated hosts than in non–vaccinated hosts at two weeks post- first vaccination (-12dpi; P<0.05) , but the peaks were accompanied by a rapid contraction by four weeks post- first vaccination. Furthermore, on exposure to pathogen challenge, the magnitude of cellular immune responses was augmented in vaccinated groups compared to the non-vaccinated groups. However, as the mock-challenged group elicited non-specific immune responses, the kinetics of cellular immunity specific to pathogen exposure in vaccinated and non-vaccinated animals was not clearly understood.

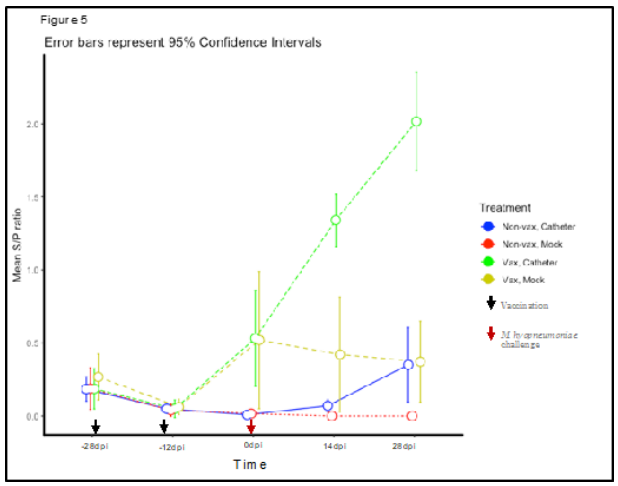
Likewise, the dynamics of humoral immune responses to *M. hyopneumoniae* vaccination and infection was determined. As shown in figure 5, the magnitude of seroconversion was higher in vaccinated animals on exposure to *M. hyopneumoniae* challenge compared to non-vaccinated infected gilts.

Similar findings have been reported with Porcine circovirus type 2 (PCV2) vaccination in pigs, although the IFNγ responses sustained longer in circulation (Ferrari et al., 2014). Furthermore, studies conducted on cellular immune responses to influenza vaccination and tetanus toxoid administration in human (Stadtmauer et al., 2011; Li Causi et al., 2015) reported comparable kinetics.

In fact, findings were in line with the normal adaptive immune responses to vaccination and secondary antigen exposure in pigs. Protective immunity comprises of two important components— the immune reactants, such as antibody or effector T cells produced in the initial infection or by vaccination, and long-lived immunological memory. The highest proliferation of circulating antigen specific effector T cells and B cells mostly occur around two weeks post-exposure and decreases subsequently as they elude from circulation to lymphoid tissues to build immunological memory. Nevertheless, the type of protection offered by antibody or effector T cells mostly depends on the 'infectious strategy and lifestyle of the pathogen' (Janeway et al., 2001).







Implications

The results indicate that *M. hyopneumoniae* vaccination induced a rapid adaptive cellular immune response as early as two weeks post-vaccination whereas humoral responses were only detected four weeks post-vaccination. Furthermore, they revealed an independent dynamic of specific T-cell and antibody responses to *M. hyopneumoniae* vaccination and infection. Nonetheless, no significant differences in cellular immune

responses between different treatment groups, post-infection was observed suggesting that timing and non-specific PMBC stimulation might have impacted our results.

Although, *M. hyopneumoniae* induced cell-mediated immunity has been associated with protection from disease, limited evidence is available for cell-mediated immunity to *M. hyopneumoniae* after vaccination. Therefore, future experiments on antigen specific T cell and B cell responses will help to better understand cellular immune function in hosts and will reveal alternative cellular assay based methods to measure the vaccine efficacy.