Environmental detection of *Mycoplasma hyopneumoniae* in breed-to-wean farms

Laura Garza-Moreno[a,b^], Carles Vilalta[b&], Maria Pieters[b,c*]

[a]*IRTA, Centre de Recerca en Sanitat Animal (CRESA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, Bellaterra, Spain*

[b] *Veterinary Population Medicine Department, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, United States*

[c]*Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, United States*

[^]*Current address: Ceva Salud Animal, Barcelona, Spain*

[&]*Current address: Upnorth Analytics, Arbeca, Lleida, Spain*

*Corresponding author: Maria Pieters. 1365 Gortner Ave St. Paul, MN 55108, United States of America. Tel: 1-612-624-7947. Email: piet0094@umn.edu

Keywords

Mycoplasma hyopneumoniae; environmental sampling; farrowing room; real-time PCR; surfaces.

Abstract

There is a need to develop cost-effective and non-invasive approaches to sample large populations to evaluate the disease status of breeding herds. In this study we assessed the presence of the *M. hyopneumoniae* genetic material in environmental surfaces and air of

farrowing rooms, and skin (udder, snout and vagina) of lactating sows at weaning, in farms having different *M. hyopneumoniae* infection status (negative, positive sub-clinically infected and positive clinically affected). *Mycoplasma hyopneumoniae* was detected in air, air deposition particles, dam and stall surfaces of the positive clinically affected herd. *Mycoplasma hyopneumoniae* could only be detected in dam and stall surfaces in sub-clinically infected herds. *Mycoplasma hyopneumoniae* was not detected in all samples collected in the negative herd. The cycle threshold of the positive PCR samples were not statistically different between sample types or farms. However, a significant difference ($p<0.05$) was observed in the percentage of positive samples between the positive clinically affected farm and the rest. Likewise, *M. hyopneumoniae* was detected in the environment and surfaces at weaning in positive breeding herds. Further testing and validation is recommended for environmental and surface samples before they can be employed as part of the *M. hyopneumoniae* diagnostic process. In addition, results from this study highlight potential sources of *M. hyopneumoniae* infection for piglets in breeding herds, especially during an outbreak.

1. Introduction

Mycoplasma hyopneumoniae (M. hyopneumoniae) is the causative agent of enzootic pneumonia (EP), one of the most important chronic respiratory diseases in the swine industry worldwide (Maes et al., 2018). Enzootic pneumonia is frequently complicated with other viral pathogens, such as *porcine reproductive and respiratory syndrome virus* (PRRSV) and/or *swine influenza A virus* (IAV), causing a more significant clinical presentation known as porcine respiratory disease complex (Pieters and Maes, 2019).

Several strategies for elimination and control of *M. hyopneumoniae* infection within herds have been proposed (Holst et al., 2015; Garza-Moreno et al., 2018; Maes et al., 2018). In consequence, various approaches are used to measure the success of the control and eradication strategies and monitoring *M. hyopneumoniae* status, such as clinical examination, lung lesion examination by abattoir surveillance and the submission of different type of samples to monitor this bacterium, mostly serum samples and respiratory swabs (Sibila et al., 2009; Pieters et al., 2017; Garza-Moreno et al., 2018).

Since the most frequent route of *M. hyopneumoniae* transmission is direct contact (nose-to-nose) from infected to susceptible pigs, especially from dam to piglets during lactation period (Nathues et al., 2014; Pieters et al., 2014), samples from lower respiratory tract are considered as the sample of choice for *M. hyopneumoniae in vivo* detection (Fablet et al., 2010). Concretely, deep tracheal catheters and laryngeal swabs have been shown as the most sensitive samples (Pieters et al., 2017; Sponheim et al., 2020). Although the reliability of both type of samples, these are sometime labor and time-consuming, because these require restraining the animal.

Other indirect transmission routes for *M. hyopneumoniae* have been also described in the literature. For instance, the bacterium has been detected in the air of barns hosting clinically experimentally infected pigs (Stark et al., 1998; Fano et al., 2005). Later studies have suggested airborne transmission to occur at long distances (Goodwin, 1984), which could reach up to 9.1 km under experimental conditions (Otake et al., 2010). However, the role of indirect transmission of *M. hyopneumoniae* via environmental contamination remains poorly understood.

A recent study showed that *M. hyopneumoniae* survived for up to 8 days at 4ºC on various surface materials commonly encountered in pig farms under experimental conditions (Browne et al., 2016). Indeed, environmental specimens such as air and surface samples are commonly used as an alternative surveillance strategy for monitoring pathogens such as PRRSV (Alonso et al., 2015; Stein et al., 2018; Vilalta et al., 2019) and IAV (Neira et al., 2016; Garrido-Mantilla et al., 2019). Nevertheless, no information is available regarding the detection and monitoring of *M. hyopneumoniae* in environmental samples under field conditions. Therefore, this study sought to detect *M. hyopneumoniae* in different types of environmental samples collected in farrowing rooms from breed-to-wean farms with different *M. hyopneumoniae* health status.

2. Materials and methods

2.1. Farms selection

Four breed-to-wean farms (A-D) with different *M. hyopneumoniae* health status, located in the Midwest United States, were conveniently selected for this investigation. Farm selection was based on prior history of *M. hyopneumoniae* infection upon consultation with the herd veterinarian. Farms were classified according to their *M. hyopneumoniae* health status (Garza-Moreno et al., 2018). Farm A was a *M. hyopneumoniae* negative farm with absence of clinical signs and record of negative samples from the last 15 years. Farms B and C were considered positive subclinical infected I and II, respectively. Farm D was classified as positive clinically affected, due to a recent *M. hyopneumoniae* outbreak confirmation by both respiratory symptomatology and detection of the bacterium in clinical specimens.

92

93 At each farm, three farrowing rooms housing suckling piglets, closest to weaning age, were
94 conveniently selected for sampling. The type and location of samples collected within each
95 room are shown in Figure 1. Briefly, air samples (AR; n=1) were collected at the most
96 central point in the room, air deposition particles (AP; n=4) from each room corner, stall
97 surface (SS; n=6) and dam surface (DS; n=6) from targeted high and low parity dams in all
98 rows of the farrowing room. Total number of collected samples at negative farm (A) varied
99 slightly (AR=3; AP=11; SS=15; DS=15) compared to other farms due its batch
100 management and negative status (Table 1).

101

102 *2.2. Environmental sampling procedures*

103 *Air and air deposition particles*

104 Air samples were obtained using an air cyclonic collector (Midwest Micro-Tek, Brookings,
105 SD, USA). Briefly, 10 mL of PBS were added to the air sampler collection vessel, which
106 was allowed to operate during 30 min, and the PBS in the collection vessel was transferred
107 into a sterile tube using a sterile pipette. Similarly, AP samples were collected using
108 aluminum foil (100 x 30 cm) placed on top of the dam stalls out from the dam's reach,
109 during 60 min. Afterwards, the foil surface was wiped with a 5 mL of a PBS impregnated
110 gauze.

111 *Dam and stall surfaces*

112 The surface of each stall and each dam was wiped with a 5 mL (8 x 8 cm) PBS impregnated
113 gauze, for SS and DS samples, respectively, which were placed in individual sterile bags.
114 For SS samples, gauze was used to swipe areas in contact with the mouth and nares of the
115 dam and its litter, such as feeders, drinkers, flooring, and stall railings. Dam surface wipes

included sampling of the nasal, udder, and vaginal areas of the dam. All samples were transported to the laboratory under refrigeration and stored at -80ºC until processing and testing.

2.3. Sample processing and testing

All samples were processed for DNA extraction by using a MagMAX™-96 Viral RNA isolation kit and MagMAX™ Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). A previous comparison performed by Vangroenweghe et al. (2015) concluded that no statistical differences were observed between the kit used in this study and the high-volume extraction method (MagMAX™-96 Pathogen RNA/DNA extraction kit), commonly used in *M. hyopneumoniae* studies. Extracted samples were tested by real-time PCR with VetMAX™ qPCR Master Mix and VetMAX™ *M. hyopneumoniae* reagents kit (Life Technologies, Grand Island, NY, USA), following manufacturer's protocol. Two different positive controls were used: 1) a commercial internal positive control (Xeno™, included in VetMax™-Plus qPCR Master Mix kit) and 2) 1) *M. hyopneumoniae strain* 232. Negative controls (PBS) were also included to assess potential contamination during extraction and PCR process. All samples were run in duplicate. Samples were considered positive for real-time PCR when cycle threshold (Ct) was equal or lower than 37, suspect if Ct values were between 37.01 and 40, and negative if undetected. Samples initially considered suspect were re-tested and classified based on the second result.

2.4. Statistical analysis

The proportion of positive samples by farm was compared using Fisher's and Chi-square tests. Comparison of Ct values between sample types and farms were performed using ANOVA and Tukey tests. The proportion of positive replicates by sample was compared using generalized linear mixed models, with farm as random effect.

Analyses were conducted using R software, version 4.0.3 (R Core Team, 2018).

3. Results

A total of 197 environmental samples were collected and tested. From the samples, 138/197 (70.1%) were collected from surfaces (DS and SS), and 59/197 (29.9%) included AR and AP. Fifteen of 69 (15/69; 21.7%) DS and 17/69 (24.6%) SS samples were detected positive for *M. hyopneumoniae*.

All AR samples (3/3; 100%) from farm D resulted positive for *M. hyopneumoniae* detection by PCR, whereas AR samples from farms A, B, and C resulted negative. Six out of 12 (50%) tested AP samples from farm D were positive to *M. hyopneumoniae*, whereas AP samples from the rest of the farms (35/47; 74.5%) were negative.

Farm D, classified as *M. hyopneumoniae* positive clinically affected, showed the highest proportion of positive environmental samples for *M. hyopneumoniae* by PCR, followed by farms C (positive subclinical infected II) and B (positive subclinical infected I; Table 1). All surface samples from farm A were negative for *M. hyopneumoniae* detection (Table 1). The proportion of positive samples from farm D was statistically significant (p value < 0.05) when compared with the other farms, either as a group or individually by sample type. No significant differences were found between sample types when the proportion was corrected by the farm (p value = 0.25).

A boxplot of the Ct values resulting from testing different environmental samples by real-time PCR in the four different farms is shown in Figure 3. Air samples showed the lowest mean Ct values (34.9±0.4) followed by DS (35.4±1.0), SS (35.5±0.9) and AP (35.8±0.7). Ct value differences observed among sample types and farms were numerical (ANOVA p value 0.515 and 0.159, respectively). Hence, the overall results were interpreted in terms of number of positive samples, rather than quantitatively. Linear models using sample type as factor and farm category as fixed or random effect were build with no significant result (data not shown).

4. Discussion

Monitoring and detection of *M. hyopneumoniae* in infected farms is important to monitor the disease and evaluate the success of control measures. Surveillance in *M. hyopneumoniae* negative farms is equally important to early detect any sign of infection and implement strategies to stop or reduce the spread of the disease. Currently, the use of samples collected from the swine lower respiratory tract, coupled with PCR testing appears to be the most prevalent and sensitive approach for *M. hyopneumoniae* detection in live pigs (Sponheim et al. 2020). Despite molecular methods as PCR demonstrate the presence of *M. hyopneumoniae* by genome detection, these are not able to evaluate viability of bacteria or possibility of infection. Moreover, pig restraint for sample collection is required, which is labor intensive and time consuming. Less invasive, less labor intensive and more cost-efficient sampling methods are needed to monitor *M. hyopneumoniae* in breeding herds. In swine barns, sampling the environment and the sow have proven useful to detect and monitor viruses such as PRRSV and IAV (Vilalta et al., 2019; Garrido-Mantilla et al., 2019). Alternative sampling methods, similar to those currently employed for detection of

187 swine viral infections, have never been tested for *M. hyopneumoniae*. Thus, in this study we
188 have assessed the use of non-invasive environmental samples for detection of *M.*
189 *hyopneumoniae* by PCR, based on the defined sow farm infection status.
190 Significant differences in the number of positive *M. hyopneumoniae* samples were observed
191 in positive clinically affected farm in which the bacterium could actively shed, compared to
192 positive subclinical infected (I and II) or negative farms. Thus, most of the environmental
193 samples in farm D yielded a *M. hyopneumoniae* PCR positive result. These observed
194 differences highlight the potential that environmental samples might have in surveillance of
195 *M. hyopneumoniae* negative herds or as a diagnostic tool when a *M. hyopneumoniae*
196 outbreak is suspected. Another interesting point is the fact that no statistical differences on
197 Ct values were observed among samples and farms. This result might be associated to the
198 small range of individual Ct values detected (from 33.86 to 36.71). Notwithstanding, it is
199 important to note that Ct values do not provide information regarding DNA quantity and,
200 consequently, it would not be possible to assume that a lower Ct value implied higher
201 bacterial pressure.
202 *Mycoplasma hyopneumoniae* was detected in the air space of ready-to-wean farrowing
203 rooms of an actively shedding farm. However, *M. hyopneumoniae* could not be detected in
204 environmental samples of sub-clinically infected herds. These results support the idea that
205 *M. hyopneumoniae* indirect transmission might be more likely to occur, especially at short
206 distances in acute infected herds, as suggested previously (Done, 1996; Goodwin, 1984;
207 Fano et al., 2005). The indirect transmission hypothesis agrees with previous research
208 where *M. hyopneumoniae* air detection was obtained only when several pigs were clinically
209 infected, and probably shedding at the same time. Stark et al. (1998) sampled the air in
210 rooms housing *M. hyopneumoniae* positive pigs under experimental and field conditions

and detected the bacterium by PCR in the air of barns in which pigs with acute clinical signs were housed. In a study evaluating the direct and indirect transmission of *M. hyopneumoniae* between pigs in the same barn, the bacterium was detected in the air shortly after pigs were infected (Fano et al., 2005). Results of other studies have shown *M. hyopneumoniae* PCR detection in the nose of farm workers (Nathues et al., 2012) and processing fluids (Vilalta et al., 2019). The most important risk factor associated to the *M. hyopneumoniae* detection by PCR in the farm workers noses was the presence of actively shedding piglets in the farm (Nathues et al., 2012). Similarly, *M. hyopneumoniae* was detected by PCR in processing fluids during a *M. hyopneumoniae* outbreak (Vilalta et al., 2020). However, *M. hyopneumoniae* was detected only during the period when pigs were actively shedding, and clinical signs could be observed, suggesting that the source of the genetic material could be the environment (Vilalta et al., 2020). Results from the above mentioned studies support the hypothesis that acutely infected pigs could shed *M. hyopneumoniae* in larger amounts than sub-clinically infected pigs, could be contaminating the air space and surfaces of the rooms in which they are housed and *M. hyopneumoniae* could be detected in the environment or other related surface.

In this investigation, different scenarios were identified when sub-clinically infected and negative herds were sampled. *Mycoplasma hyopneumoniae* prevalence in sub-clinically infected and negative herds was lower than in recently infected farms. Thus, *M. hyopneumoniae* was not detected in the air, but was detected in a lower percentage of samples from surfaces related or close to pigs in sub-clinically infected herds than in the herd that was actively shedding. In the case of the negative herd, *M. hyopneumoniae* was not detected in any of the environmental samples tested. However, it is important to note that the overall accuracy of *M. hyopneumoniae* detection in the environment, compared to

other samples used as reference, such as laryngeal swabs or deep tracheal catheters, is unknown. Moreover, the small sample size used in this study could lead to underestimating the true proportion of *M. hyopneumoniae* positive litters in both, clinically infected and negative herds, given that the bacterial prevalence could have been very low. A larger investigation, including a greater number of swine farms and sampling events can be suggested to confirm the results of this study. Repetitive sampling could help to define whether the percentage of positive and negative samples was consistent over time in negative and subclinically infected herds, and if the percentage of positive samples would have decreased in the active shedding herd. Furthermore, studies are needed to elucidate the sensitivity of the environmental samples compared to the clinical specimens used as reference and their potential for *M. hyopneumoniae* detection under different scenarios.

Prior work investigating the use of udder skin wipes and surface wipes for detection of PRRSV identified that the probability to detect a positive sample increased when the proportion of PRRSV positive pigs in the litter were higher and had greater viral load in serum (Vilalta et al., 2019). The overall PRRSV sensitivity of udder skin wipes and surface wipes at the litter level compared with serum was 43% and 47%, respectively. However, the accuracy of IAV detection with the use of udder skin wipes was greater than for PRRSV. Results from a study assessing different sampling methodologies detected that udder skin wipes were the optimum sample to detect and isolate IAV positive piglets together with oropharyngeal swabs (Garrido-Mantilla et al., 2019). Collecting laryngeal swabs or deep tracheal catheter samples from dams and piglets from each farrowing stall in the room would have allowed to estimate the status of the litter and the prevalence of the herd. Further studies should address the sensitivity of sampling the farrowing stall and the

pig and under different prevalence scenarios, even its potential use to evaluate the environmental contamination regarding other swine Mycoplasmas as *Mycoplasma hyorinis and Mycoplasma hyosynoviae*.

In this study, the detection of *M. hyopneumoniae* in pig surfaces or in close contact within the farrowing room have been documented. It is important to note that the origin of the bacterium that was detected by PCR remains unknown, as pigs and/or environment could have been infected. The presence of *M. hyopneumoniae* in surfaces is relevant for different reasons. First, dams that yielded a positive result on their surfaces (snout, udder skin and vagina) could potentially transmit the bacterium to their own or adopted piglets. Secondly, contaminated surfaces could be a source of the bacterium and potentially infect susceptible pigs. Studies evaluating the role of surfaces as an indirect source of *M. hyopneumoniae* to piglets have not been reported in the literature. Other studies on PRRSV virus, have pointed at the importance of correct cleaning and disinfection of the surfaces that are in contact with susceptible pigs (Dee et al., 2004). Further studies should be conducted in order to address and quantify the role of farrowing rooms on *M. hyopneumoniae* infections, as piglet colonization with the bacterium occurs in this facility. Nevertheless, it seems relevant to emphasize the implementation of strict biosecurity measures that could limit the indirect transmission by air and contaminated surfaces, especially in farms working towards *M. hyopneumoniae* elimination.

Another important limitation to be considered is that the number of rooms were conveniently selected. Convenience sample has its advantages and disadvantages. While it can be very useful to collect cost-effective and preliminary data, it can also lead to bias and have lack of power to generate conclusions. Thus, results from this study should be taken

carefully as they might be biased due to the small sample size. However, the information generated her will be beneficial to calculate a sample size in similar studies.

In conclusion, this study provides information on *M. hyopneumoniae* environmental sampling in herds with different *M. hyopneumoniae* status. Our findings suggest that sampling the environment could be a complementary, quick, and pig welfare friendly option compared to conventional laryngeal or deep tracheal swabs in the face of a *M. hyopneumoniae* outbreak. Environmental samples are easy to collect and do not require any additional training. Furthermore, this study highlights the role that the environment and dams could have in the transmission and maintenance of the disease in the farrowing room.

Conflicts of interest

The authors declare no conflict of interest. This study did not include the use or evaluation of commercial products.

Funding

This work was funded by the Mycoplasma Research Laboratory at the University of Minnesota, the Minnesota Agricultural Experiment Station and the Minnesota Department of Agriculture, under Rapid Agricultural Response Fund project #RR251.

Acknowledgments

The authors would like to thank Mark Schwartz, Drs. Samuel Baidoo, Mark Wagner, Laura Bruner, Matt Allerson, and the farm personnel who helped with this investigation.

References

Alonso, C., Raynor, P.C., Davies, P.R., Torremorell, M., 2015. Concentration, Size Distribution, and Infectivity of Airborne Particles Carrying Swine Viruses. PLoS ONE 10(8): e0135675. doi:10.1371/journal.pone.0135675

Browne, C., Loeffler, A., Holt, H.R., Chang, Y.M., Lloyd, D.H., Nevel, A., 2016. Low temperature and dust favour *in vitro* survival of *Mycoplasma hyopneumoniae* : time to revisit indirect transmission in pig housing. Lett. Appl. Microbiol. 1–6. doi:10.1111/lam.12689

Dee, S., Deen, J., Otake, S., Pijoan, C., 2004. An experimental model to evaluate the role of transport vehicles as a source of transmission of porcine reproductive and respiratory syndrome virus to susceptible pigs. Can J Vet Res; 68 (2):128-133.

Done, S., 1996. Enzootic pneumonia (mycoplasmosis) revisited. Pig Journal 38, 40-61

Fablet, C., Marois, C., Kobisch, M., Madec, F., Rose, N., 2010. Estimation of the sensitivity of four sampling methods for Mycoplasma hyopneumoniae detection in live pigs using a Bayesian approach. Vet. Microbiol. 143, 238–245. doi:10.1016/j.vetmic.2009.12.001

Fano, E., Pijoan, C., Dee, S., 2005. Evaluation of the aerosol transmission of a mixed infection of Mycoplasma hyopneumoniae and porcine reproductive and respiratory syndrome virus. Vet Rec. 23;157(4):105-8. doi: 10.1136/vr.157.4.105.

Garrido-Mantilla, J., Alvarez, J., Culhane, M., Nirmala, J., Cano, J.P., Torremorell, M., 2019. Comparison of individual, group and environmental sampling strategies to conduct influenza surveillance in pigs. BMC Vet. Res. 15, 1–10. doi:10.1186/s12917-019-1805-0

Garza-Moreno, L., Segalés, J., Pieters, M., Romagosa, A., Sibila, M., 2018. Acclimation

328 strategies in gilts to control Mycoplasma hyopneumoniae infection. Vet. Microbiol.
329 219, 23–29. doi:10.1016/j.vetmic.2018.04.005
330 Goodwin, R., 1984. Apparent infection of enzootic-pneumonia-free pig herds: early signs
331 and incubation period. Vet Record 115, 320-324
332 Holst, S., Yeske, P., Pieters, M., 2015. Elimination of Mycoplasma hyopneumoniae from
333 breed-to-wean farms: A review of current protocols with emphasis on herd closure and
334 medication. J Swine Heal. Prod 23, 321–330.
335 Maes, Sibila, M., Kuhnert, P., Segales, J., Haesebrouck, F., Pieters, M., 2018. Update on
336 Mycoplasma hyopneumoniae infections in pigs : Knowledge gaps for improved
337 disease control. Transbound. Emerg. Dis. 1–15. doi:10.1111/tbed.12677
338 Nathues, H., Chang, Y.M., Wieland, B., Rechter, G., Spergser, J., Rosengarten, R.,
339 Kreienbrock, L., grosse Beilage, E., 2014. Herd-Level risk factors for the
340 seropositivity to mycoplasma hyopneumoniae and the occurrence of enzootic
341 pneumonia among fattening pigs in areas of endemic infection and high pig density.
342 Transbound. Emerg. Dis. 61, 316–328. doi:10.1111/tbed.12033
343 Nathues, H., Woeste, H., Doehring, S., Fahrion, AS., Doherr, MG., Beilage, EG., 2012.
344 Detection of Mycoplasma hyopneumoniae in nasal swabs sampled from pig
345 farmersVeterinary Record 170, 623.
346 Neira, V., Rabinowitz, P., Rendahl, A., Paccha, B., Gibbs, S.G., 2016. Characterization of
347 Viral Load , Viability and Persistence of Influenza A Virus in Air and on Surfaces of
348 Swine Production Facilities. PLoS One 11, 1–11. doi:10.1371/journal.pone.0146616
349 Otake, S., Dee, S., Corzo, C., Oliveira, S., Deen, J., 2010. Long-distance airborne transport
350 of infectious PRRSV and Mycoplasma hyopneumoniae from a swine population
351 infected with multiple viral variants. Vet. Microbiol. 145, 198–208.

352 doi:10.1016/j.vetmic.2010.03.028
353 Pieters, M., Cline, G.S., Payne, B.J., Prado, C., Ertl, J.R., Rendahl, A.K., 2014. Intra-farm
354 risk factors for Mycoplasma hyopneumoniae colonization at weaning age. Vet.
355 Microbiol. 172, 575–580. doi:10.1016/j.vetmic.2014.05.027
356 Pieters, M., Daniels, J., Rovira, A., 2017. Comparison of sample types and diagnostic
357 methods for in vivo detection of Mycoplasma hyopneumoniae during early stages of
358 infection. Vet. Microbiol. 203, 103–109. doi:10.1016/j.vetmic.2017.02.014
359 Pieters, M., Maes, D., 2019. Mycoplasmosis, Eleventh Edit. ed, Diseases of Swine
360 R Core Team, 2018. R a language and environment for statistical computing.
361 Sibila, M., Pieters, M., Molitor, T., Maes, D., Haesebrouck, F., Segalés, J., 2009. Current
362 perspectives on the diagnosis and epidemiology of Mycoplasma hyopneumoniae
363 infection. Vet. J. 181, 221–231. doi:10.1016/j.tvjl.2008.02.020
364 Sponheim, A., Alvarez, J., Fano, E., Schmaling, E., Dee, S., Hanson, D., Wetzell, T.,
365 Pieters, M., 2020. Comparison of the sensitivity of laryngeal swabs and deep tracheal
366 catheters for detection of Mycoplasma hyopneumoniae in experimentally and naturally
367 infected pigs early and late after infection. Vet. Microbiol. 241, 108500.
368 doi:https://doi.org/10.1016/j.vetmic.2019.108500
369 Stark, K.D.e., 1998. Systems for the prevention and control of infectious diseases in pigs.
370 PhD thesis, Massey University, Palmerston North, New Zealand..
371 https://www.massey.ac.nz/massey/fms/Colleges/College%20of%20Sciences/Epicenter
372 /docs/KatharinaStarkPhD.pdf?0F604B246DB6A3B204BA7D811D47FB9D (last
373 accessed: 07/09/2020).
374 Stein, H., Schulz, J., Morgenstern, R., Voglmayr, T., Freymüller, G., Sinn, L., Rümenapf,
375 T., Hennig-pauka, I., Ladinig, A., 2018. PRRSV-1 under Experimental and Field

Conditions. Animals 8. doi:10.3390/ani8120233

Vangroenweghe F, Karriker L, Main R, et al. Assessment of litter prevalence of *Mycoplasma hyopneumoniae* in preweaned piglets utilizing an antemortem tracheobronchial mucus collection technique and a real-time polymerase chain reaction assay. *Journal of Veterinary Diagnostic Investigation*. 2015;27(5):606-610. doi:10.1177/1040638715595062

Vilalta, C., Sanhueza, J., Garrido, J., Murray, D., Morrison, R., Corzo, A., Torremorell, M., 2019. Indirect assessment of porcine reproductive and respiratory syndrome virus status in pigs prior to weaning by sampling sows and the environment. Vet. Microbiol. Volume 237, 108406. doi: https://doi.org/10.1016/j.vetmic.2019.108406

Vilalta, C., Garcia-Morante, B., Sanhueza, JM., Schwartz, M., Pieters, M., 2020. PCR detection of *Mycoplasma hyopneumoniae* in piglet processing fluids in the event of a clinical respiratory disease outbreak. Vet Record Case Reports 8: e001045. doi: 10.1136/vetreccr-2019-001045

Figure legends

Figure 1. Schematic representation of environmental sampling location in farrowing rooms.

AR: Air samples; AP: Air deposition particles; DS: Dam surface; SS: Stall surface.

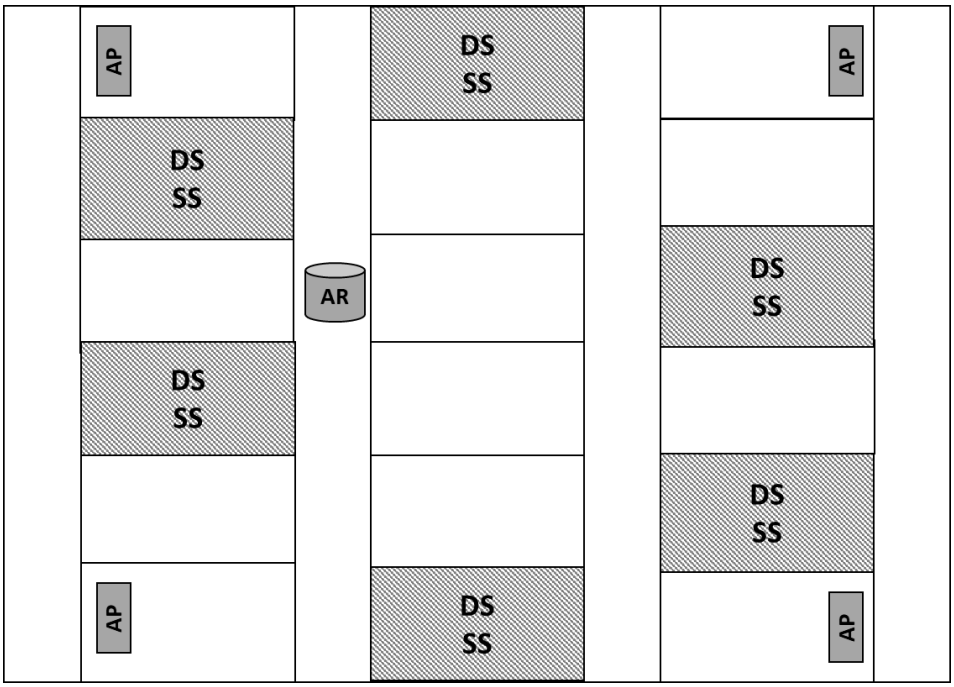
Samples were collected in three different rooms at each breed-to-wean farm.

Figure 2. Cycle threshold values from individual environmental *Mycoplasma hyopneumoniae* positive samples (Ct value < 37) in three positive farms (B, C and D). AP: Air deposition particles; AR: Air; DS: Dam surface; SS: Stall surface. The label indicates the number of positive samples in each category.

Table 1. Proportion and percentage of *Mycoplasma hyopneumoniae* positive samples tested in four breed-to-wean farms with different *M. hyopneumoniae* health status.

Herd ID	*M. hyopneumoniae* health status	Environmental samples *				Total
		Dam surface (DS)	Stall surface (SS)	Air samples (AR)	Air deposition particles (AP)	
A	Negative	0/15 (0.0%)	0/15 (0.0%)	0/3 (0.0%)	0/11 (0.0%)	0/44 (0.0%)
B	Positive subclinical infected I	1/18 (5.5%)	1/18 (5.5%)	0/3 (0.0%)	0/12 (0.0%)	2/51 (3.9%)
C	Positive subclinical infected II	2/18 (11.1%)	1/18 (5.6%)	0/3 (0.0%)	0/12 (0.0%)	3/51 (5.9%)
D	Positive clinically affected	12/18 (66.6%)	15/18 (83.3%)	3/3 (100%)	6/12 (50.0%)	36/51 (70.6%)
Total	-	15/69 (21.7%)	17/69 (24.6%)	3/12 (25.0%)	6/47 (12.8%)	-

* Tested for *Mycoplasma hyopneumoniae* by real-time PCR.



AR: Air; AP: Air deposition particles; DS: Dam surface; SS: Stall surface.

Figure 1. Representation of environmental sampling location per each farrowing room (x3).

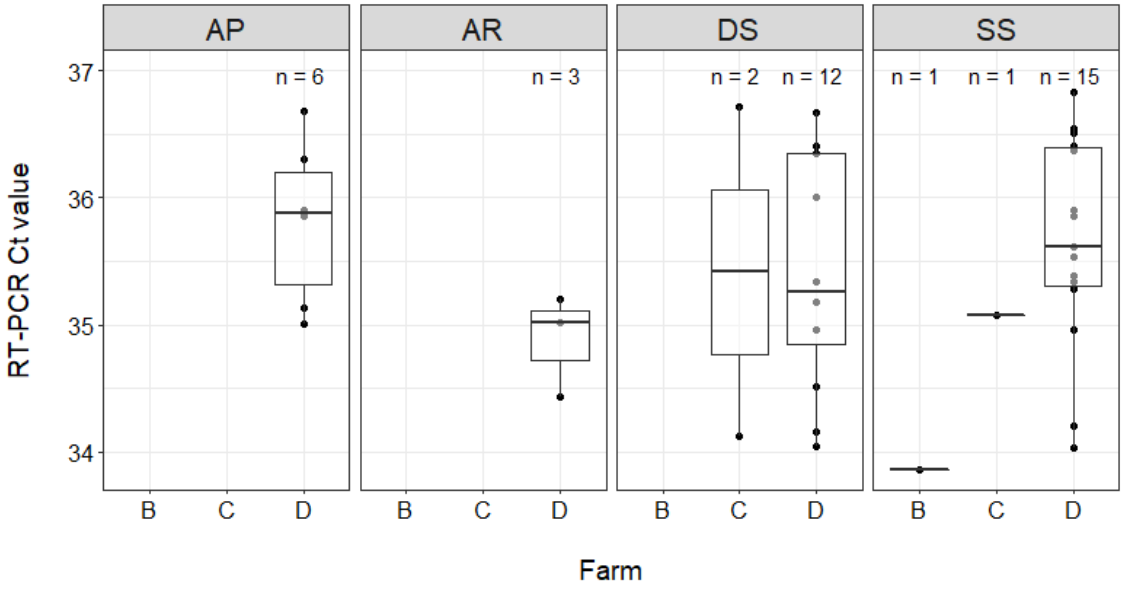


Figure 2. Whiskers and boxplot of the cycle threshold (Ct) values from individual environmental *Mycoplasma hyopneumoniae* positive (Ct<37) samples in three positive farms (B, C and D). AP: Air deposition particles; AR: Air; DS: Dam surface; SS: Stall surface. The label indicates the number of positive samples in each category.