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Profiling *Mycoplasma hyopneumoniae* in farms using serology and a nested PCR technique

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Summary

Objective: To profile herds experiencing *Mycoplasma hyopneumoniae* pneumonia using both a nested polymerase chain reaction (PCR) technique and serology and to compare both techniques.

Methods: Five commercial farms with a history of mycoplasmal pneumonia participated in the study. Samples were taken from different age groups during a single visit, starting at 5, 6, 7, or 8 weeks of age and up to nearly marketing age. Ten animals per age group (five different age groups per farm) were randomly selected and nasal swabs and blood samples taken. DNA from nasal swabs was extracted and the nested PCR to detect *M. hyopneumoniae* infected animals was performed. Sera were tested for antibodies to *M. hyopneumoniae*. The estimated antigen and antibody prevalence per age group was calculated, and plotted with their corresponding confidence intervals. Differences between both tests were measured using χ^2 analysis.

Results: Serum and bacterium profiles for each farm were obtained. The nested PCR showed a higher proportion of infected animals in the early spread of the microorganism than serology, and provided more accurate information on the dynamics of infection.

Implications: The nested PCR detects *M. hyopneumoniae* earlier then serology. Moreover, detecting *M. hyopneumoniae* from nasal swabs offers new and valuable information in helping interpret serum profiles and figuring out when animals get infected and how and where *M. hyopneumoniae* is being transmitted. This information will potentially allow for better timing in the application of medication, vaccination, or management strategies.

Keywords: swine, *M. hyopneumoniae*, nested PCR, serum profiles, bacterium profiles.

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Mycoplasma hyopneumoniae is one pathogen involved in enzootic pneumonia.[1] Although control of the disease is possible through vaccination,[2] medication,[2] or management strategies,[3,4] timing of these treatments is critical for their effectiveness.

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Until recently, serological tests were the only commonly available diagnostic tools to detect exposure to microorganism in live animals and to obtain a better understanding of the dynamics of the disease (Sitjar M, et al. *Proc IPVS Cong*, 1994;p133). However, these serological tests have some limitations and are difficult to interpret. Serology detects the onset of seroconversion, not the onset of infection. Time to seroconversion after exposure to *M. hyopneumoniae* varies. In several experimental challenge studies, antibodies were detected at 2 weeks postinfection by ELISA in the first animals that seroconverted.[5–7] However, not all animals seroconverted at the same time, and it was not until 4–5 weeks after challenge that all animals had detectable serum antibodies. In contact-exposed piglets, antibodies are usually detected later. In a study where contact pigs were exposed to intranasally infected pigs, pigs with antibodies were initially seen 5 weeks after contact, and it was not until 9 weeks after contact that all animals had seroconverted.[8] In field infections, time of seroconversion appears also to be delayed. In a study where antibodies against *M. hyopneumoniae* from several groups were serially measured and the thorax of each pig radiographed, it was concluded that the peak severity of lung lesions was associated with seroconversion, although the latter was delayed approximately 6–8 weeks.[9]

At present, the ELISA is considered the most useful test for serology. It can detect all classes of immunoglobulins, gives quantitatively measurable results, and is very sensitive. When first developed,[10] the ELISA contained protein antigens from sodium dodecyl sulfate (SDS)-solubilized *M. hyopneumoniae* cells, which resulted in nonspecific reactions.[11] The specificity of the ELISA has been improved[12–14] by using the neutral detergent Tween 20 for extraction of membrane proteins from *M. hyopneumoniae* whole cells, thereby reducing the content of high (> 90 kD) and low (<31 kD) molecular weight proteins.[15] It has been shown[14] that cross reactions with *Mycoplasma flocculare* are minimal, especially in natural infections, although they still exist. Therefore, the results of the Tween 20 ELISA must be interpreted with caution, especially when the optical densities are between 0.200 and 0.260. These readings can be observed in early or late infections, but could also be due to cross reactions with *M. flocculare.*

Recently, a nested PCR that is able to detect *M. hyopneumoniae* from nasal swabs has been described.[16] Other one-step PCRs for *M. hyopneumoniae* have been described,[17–20] but they are apparently not able to consistently detect the microorganism from nasal swabs. The primers have been tested with most mycoplasmas and acholeplasmas

described in swine (*Mycoplasma hyorhinis, Mycoplasma flocculare, Mycoplasma hyosynoviae, Mycoplasma hyopharyngis Mycoplasma buccale, Actinobacillus axanthum, Actinobacillus granularum, Actinobacillus laidlawii*) as well as several avian mycoplasmas. The primers of the PCR for *M. hyopneumoniae* did not amplify DNA from any of these microorganisms.

The primers have been also tested in live animals, in a challenge study using *M. hyopneumoniae*–free pigs. Before challenge, all animals were swabbed and shown to be mycoplasma negative by PCR.[16] Although PCR is a very promising tool to profile herds to determine the carrier state of incoming gilts and establish the health status of different farms from which pigs are going to be commingled, the significance of obtaining a positive result from a nasal swab is still unknown.

The objective of this study was to profile five herds experiencing respiratory disease related with mycoplasma using the nested PCR technique and serology.

Materials and methods

Farms

Five farms with an ongoing *M. hyopneumoniae* disease problem, as diagnosed by the veterinarian of the farm, were selected for the study:

- Farm A was a three-site production system, with all-in–all-out (AIAO) management by room in the finishers. The sow herd was negative to *M. hyopneumoniae* according to serology and history. The mycoplasma outbreak was seen in the finishing barns. No vaccination nor medication for *M. hyopneumoniae* was being used.
- Farm B was a 1200-sow herd, two-site production system, with wean-to-finish buildings managed AIAO by building. The pigs started coughing at around 16 weeks of age, and continued until they went to market. A vaccination protocol with M+Pac™ *M. hyopneumoniae* bacterin (Schering Plough; Madison, New Jersey) was being used. A 1-mL dose was administered subcutaneously at 5 and 7 weeks of age.
- Farm C was a three-site production system with piglets commingled from ten different sow herds, each of them containing 2000 head. The nurseries and finishers were managed AIAO by site. Weaning age was 19 days on average. The mycoplasma-related cough was seen 50 days after entering the finishers (around 16 weeks of age). No vaccines against *M. hyopneumoniae* were being used.
- Farm D was a 6000-sow herd, three-site production system that was managed AIAO by building and that weaned at 16–17 days of age. Respiratory signs were seen at around 18 weeks of age. A vaccination protocol with Suvaxyn Respifend™ (Fort Dodge; Fort Dodge, Iowa) was being used: the first 2-mL dose was administered intramuscularly (IM) at 7 weeks of age and the second one at 9 weeks.
- Farm E was a three-site production system. Weaned pigs were commingled in the nurseries from 10 different sow herds, each of them with 2500 head. Nurseries and finishers were managed AIAO by site. Slight respiratory clinical signs, affecting approximately 10%–20% of the pigs, were seen by the end of the finishing stage. No vaccination or medication protocols were being used.

Samples

Samples were taken from different age groups during a single visit, starting with nursery-aged pigs (approximately 6 weeks of age) and then sampling at 4-week age intervals(at 6, 10, 14, 18, and 22 weeks of age). Ten animals per age group were restrained with a snare and nasal swabs and blood samples were taken. Nasal samples were taken using swabs with transport media (Culturette™, Becton Dickinson). The swab was introduced into the nostrils, reaching deeply into the turbinates. Nasal swabs were kept moistened with the transport media and sent overnight on ice to the laboratory, where they were further processed.

Culture was not attempted because it lacks sensitivity in nasal swab samples[17] and is more time consuming than the nested PCR. Blood samples were used for serology and nasal swabs for detecting the microorganism with the nested PCR. DNA extracted from a pure culture of *M. hyopneumoniae* was used as positive control and double-distilled water submitted to the same extraction protocol as negative control.

Serology

Serology samples were sent to Iowa State University Diagnostic Laboratory, where they were assayed with the Tween 20 ELISA, as previously described.[16] This test does not differentiate vaccinated from infected animals.

DNA extraction

Nasal swabs were resuspended in 350 mL of sterile PBS. DNA was extracted as previously described.[18] Briefly, suspensions were boiled for 5 minutes, DNA was extracted with phenol:chloroform, and precipitated with ethanol and sodium acetate (pH 5.2, final concentration of 0.5M). DNA was resuspended in 40 mL of double-distilled water.

Nested PCR

Polymerase chain reaction was performed using primers and conditions previously described.[18] Two sets of primers from the 16S ribosomal gene were used. The outer primer pair consisted of:

- a forward primer: 5´- GAG CCT TCA AGC TTC ACC AAG A- 3´(nucleotide positions 212–233), and
- a reverse primer: 5´ - TGT GTT AGT GAC TTT TGC CAC C- 3´ (nucleotide positions 839–860).

The inner primer pair consisted of:

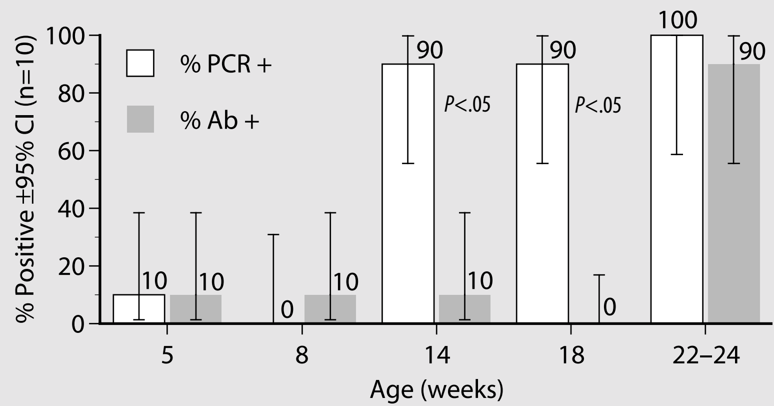
- a forward primer: 5'´– ACT AGA TAG GAA ATG CTC TAG T-3´ (nucleotide positions 463–484), and
- a reverse primer: 5´- GTG GAC TAC CAG GGT ATC T-3´ (nucleotide positions 797–815).

Five µL of the DNA preparation were used as PCR templates. Five µL of the DNA preparation were used as PCR templates in the first reaction and 0.5 µL of the product were used for the second reaction. The amplifications were performed in a 25-µL reaction mixture containing 0.2 mM concentration of each primer, 20 pmol of each nucleotide (Boehringer Mannheim; Germany), 1×PCR- buffer (Boehringer

Mannheim), 5% glycerol, 3mM $MgCl_2$ (Boehringer Mannheim), and 1U of TaqDNA polymerase (Boehringer Mannheim). Both reactions where performed in a thermocycler (GeneAmp PCR system 2400, Perkin-Elmer; Branchburg, New Jersey) and required the same conditions: 30 cycles, denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 30 seconds.

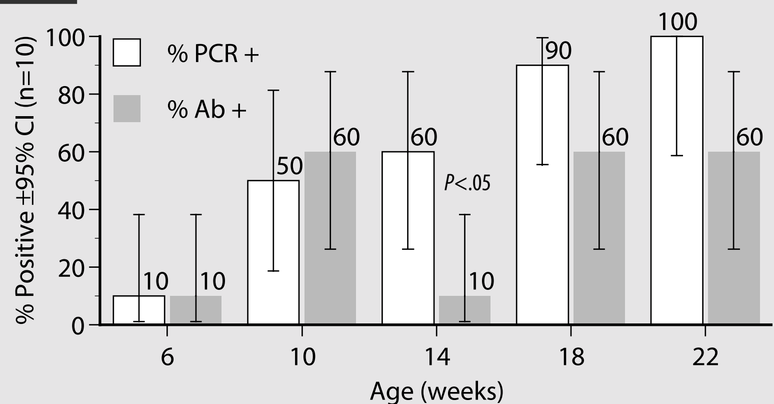
Seven-µL aliquots of the amplified samples were analyzed by electrophoresis in a 1% agarose gel with 0.5 mg per mL of ethidium bromide, and then visualized and photographed (Eagle Eye, Stratagene; La Jolla, California).

Figure 1



Farm A: serum profiles and nested PCR results.

Figure 2



Farm B: serum profiles and nested PCR results.

Analysis of results

Individual samples were tested separately, but because animals were not identified, the serology and nested PCR results of each individual animal could not be determined.

χ^2 analysis was used to compare the proportion of pigs that were positive to the PCR test versus the serologic test in each farm, at each sampling period. The data on the individual farms were also collapsed and the overall differences between both tests evaluated. The latter was done by comparing the tests over time using a stratified analysis, Mantel-Haenszel χ^2, as well as at each specific sampling period with a χ^2 analysis. Odds ratios were calculated using the Mantel-Haenszel procedure.

Results

Farm A

The nurseries appeared to have a low prevalence of *M. hyopneumoniae,* both by PCR and serology (Figure 1). There were no statistical differences detected in the proportion of positive pigs between the two tests until the 14-week age group. When pigs entered the finishers they rapidly became infected: the 14-week-old group already showed 90% of positive animals by PCR, and a high percentage of positive animals was also observed in the 18-week-old group. Seroconversion was not detected until much later, in the 22- to 24- week-old group, where a high proportion of PCR- and serology-positive animals were observed.

Farm B

Piglets entered the wean-to-finish barn with only a few animals positive to *M. hyopneumoniae* by the nested PCR (Figure 2). The number of PCR-positive pigs steadily increased with age, until a critical mass of infected pigs was reached and mycoplasma-related coughing appeared. The antibodies detected until 14 weeks of age were probably due to vaccination, and seroconversion due to infection was not detected until the 18-week-old group.

Farm C

Nursery pigs seemed to have a low prevalence of *M. hyopneumoniae*, which could only be detected by PCR (Figure 3). An increasing proportion of PCR-positive animals were detected over time, reaching a peak at 16 weeks of age, which coincided with the onset of cough. In the finishers, the proportion of positive animals detected by PCR was greater ($P<.05$) than the one obtained with serology, until the 20-week-old group. In the latter group seroconversion was detected for the first time, and the proportion of serology-positive animals was greater than the PCR positive animals ($P<.05$) in the 24-week-old group.

Farm D

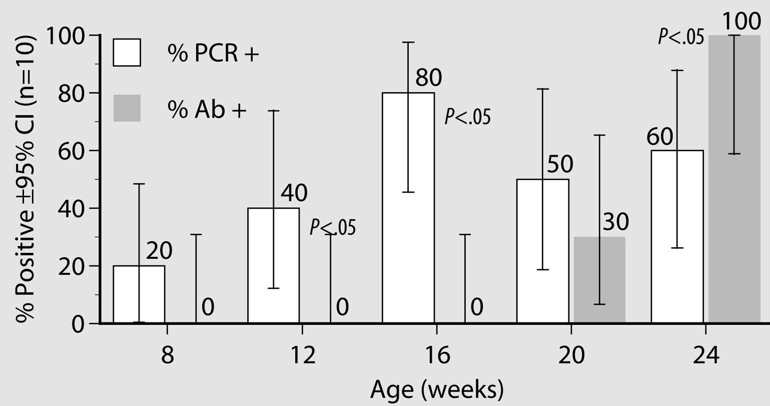
Pigs in the nurseries had relatively large numbers of positive animals, which kept increasing in the finisher, where mycoplasma-related coughing appeared (Figure 4). The antibodies detected at 10 weeks of age were probably due to vaccination. Antibodies due to *M. hyopneumoniae* infection appeared at 18 weeks of age.

Farm E

A few nursery pigs were positive to *M. hyopneumoniae* PCR (Figure 5). When the pigs entered the finishers, the proportion of PCR-positive pigs remained at relatively low numbers. From 18–24 weeks of age, an abrupt increase in the detection of *M. hyopneumoniae* in nasal swabs occurred, which coincided with the appearance of mycoplasma-related cough. Increasing antibody titers were observed at 18 and 24 weeks of age. Although PCR detected equal or greater proportion of positive pigs than serology in all sampled groups, significant differences between serology and PCR where only observed in the 24-week-old group.

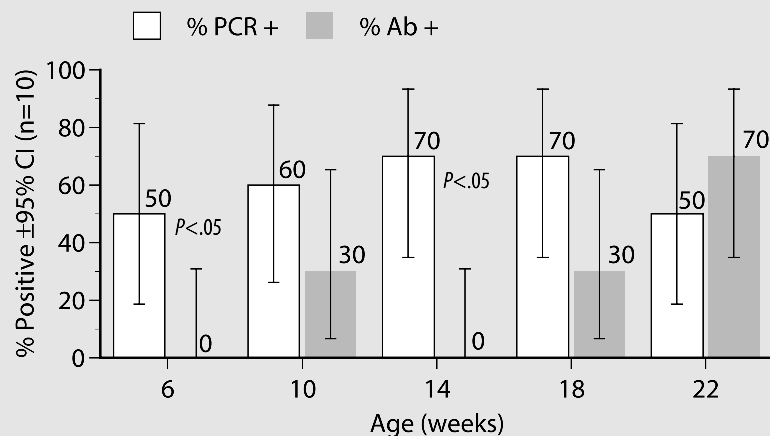
Across all five herds, the nested PCR was able to detect more positive animals than serology in the 14- to 16-week-old and 18- to 20-week-old groups (Figure 6). A larger proportion of PCR-positive versus serology-positive animals was also detected in the first two age groups in the finishers ($P<.05$). The chance of finding a positive animal by PCR was 27.5 times higher than using serology in the 14- to 16-week age group, and five times higher in the 18–20 week age group. In the 14- to 16-week-old group, the odds ratio of the PCR-positive versus serology-positive was 27.85, and in the 18- to 20-week-old group the odds ratio was 5.17. In the oldest age group, no differences were detected between serology and PCR ($P>.05$). Overall, there was a 3.44 times greater chance of finding a positive animal with the nested PCR technique than with serology.

Figure 3



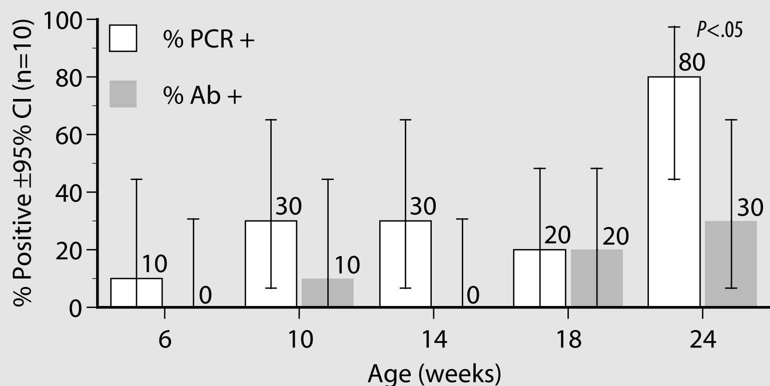
Farm C: serum profiles and nested PCR results.

Figure 4



Farm D: serum profiles and nested PCR results.

Figure 5



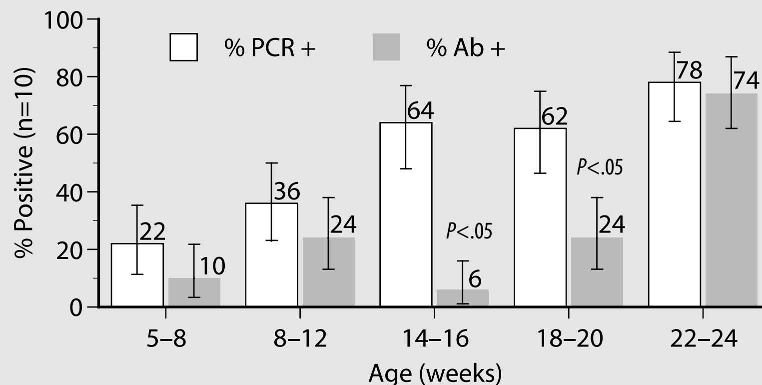
Farm E: serum profiles and nested PCR results.

Discussion

This study was conducted in order to compare the nested PCR technique with serology in the profiling of herds. Traditionally, serum profiles have used 10–15 animals of different age groups.[9,22,23] Although these sample sizes are not sufficient to make an accurate estimate of the prevalence of positive animals in each age group, a rough estimate of the dynamics of infection can be drawn. The sample size required to estimate a prevalence varies with the expected prevalence, the level of confidence, and the desired accuracy. If either low or high prevalences are expected, 25 animals suffice to obtain an estimate with a 90% confidence interval and an accuracy of 10%. When the expected prevalence approaches 50%, larger samples should be taken: approximately 70 animals. Because of this, traditional seroprofiles, as well as the PCR profiles performed in this study, should not be used to estimate prevalence. They are, however, very useful for obtaining a picture of the farm's disease dynamics that can be used to establish control strategies.

This study showed that the nested PCR is a valuable technique in profiling farms with *M. hyopneumoniae*. It was found that the proportion of the pigs testing positive via PCR was higher than those found by serology, specially during the early phase of the spread of the

Figure 6



Summary of the serum profiles and nested PCR results of all farms. Each age group contained 50 animals.

organism. However, no statistical differences were found between PCR and serology in the nursery groups, presumably because of the antibodies detected in the vaccinated groups, the low prevalence of positive animals, and the small sample size used in the study. In older animals (22–24 weeks of age), a larger proportion of animals had seroconverted, and therefore PCR and serology did not differ significantly.

Detecting the microorganism has some advantages over serology: dynamics of infection are better evaluated, and therefore more accurate information can be obtained regarding when the animals get exposed to the microorganism. This information will allow the best timing for administering medication, vaccination, and/or management strategies. As seen in this study, time to seroconversion appeared to be variable: in Farm E, low mycoplasma prevalence was found until the 24-week group, where 80% of the animals tested PCR positive and 30% were serologically positive. This increase coincided with the appearance of respiratory clinical signs. In Farm C, there was a large increase in PCR-positive animals between the 12- and 16-week-old groups. Clinical signs appeared at 16 weeks of age but seroconversion was not detected until 4 weeks later. The low prevalence of positive animals in the nurseries may have proven insufficient to trigger clinical signs or seroconversion in this farm, and mycoplasma-related cough was not evident until a large percentage of animals were PCR positive. In Farm A, there was a sharp increase of positive animals from 8–14 weeks of age, but seroconversion was not detected until the 22–24 week age group. This apparent delay of seroconversion in naturally infected pigs has been previously described, and different intervals have been reported, ranging from 4 weeks to 9 weeks following contact exposure.[9–11] Because of this variability, it is difficult to determine time of infection from serology. The variability may be a measure of infection dose, since this dose appears to play a role in lesion severity and amount of shedding by the infected animal[9] and therefore probably also in the time of seroconversion of contact pigs.

The slower spread of the microorganism does not explain entirely the delay of seroconversion detected in field cases or contact exposed animals. Clinical signs and pneumonic lesions have been found to be associated.[24] Studies have shown that the interval from onset of clinical signs or peak pneumonic lesions to seroconversion is increased in field cases compared to what is found in experimental challenge experiments.[11,24] In an intratracheal experimental infection, coughing started at 2 weeks postinfection and peaked at 5 weeks, and seroconversion was detected at 3–4 weeks after infection—2 weeks after the coughing began.[24] Conversely, in a field study from a farm infected with *M. hyopneumoniae,* seroconversion was not detected until 6–8 weeks after the onset of peak pneumonia.[11] Also, in that latter study, a trend to earlier seroconversion was observed when the pneumonic peak was higher. Therefore, magnitude of peak appears to affect not only the magnitude of seroconversion, but also the time to seroconversion after infection.

Because of the delay and variability of seroconversion after infection, it is difficult to extrapolate the moment of infection from the serology results. The nested PCR could be used to assist in determining the moment of infection and therefore to optimize the timing of the vaccination and medication protocols.

The two vaccinated herds showed increasing, and then later decreasing antibody titers shortly after vaccination. However, the proportion of positive pigs was not as high as expected, and the decay and disappearance of antibodies was rapid. This could be due to the nature of the bacterins used, the serological test, or inadequate administration or timing of the vaccines. However, this does not imply that the vaccines were ineffective, since the relationship between antibodies and protective immune response against *M. hyopneumoniae* is still under debate. Some researchers have suggested that the humoral immune response is protective,[25,26] whereas others have proposed that it is the cellular immune response.[27] It was also observed that vaccination did not prevent presence of *M. hyopneumoniae* in the nose, as detected by PCR from nasal swabs. This is in accordance with other studies, where vaccination did not prevent the isolation of *M. hyopneumoniae* from pigs.[2] However, no conclusions regarding the efficacy of the vaccines could be drawn, because no unvaccinated control groups were available in the vaccinated farms.

An important limitation of this study is that it was a cross-sectional study, not a prospective study. Therefore, the profiles obtained do not show the evolution of a same group of pigs during their life, but the status of disease in each age group at a point in time. This has the disadvantage of not always reflecting the evolution of infection rate with age. In the case of stable herds, these studies are fairly adequate in showing the evolution of disease throughout the production system. This is not the case, however, in unstable herds, or in offsite herds,

where each group is different and disease varies with each group.

The nested PCR also has some limitations: it is a qualitative, not quantitative test. The nested PCR will give a positive result if there are at least 80 microorganisms in the sample.[16] In this study, only the number of positive animals in a particular age group was detected, but the bacterial load that these animals were carrying could not be determined. This latter information can be important in assessing the potential of these animals to spread disease, and establishing the significance of finding such a positive animal. However, if the organism is detected, even if it is present only in low numbers, there is a risk that the animal can be shedding and will infect other animals. A quantitative test would better explain and/or predict the slower or more rapid spread of mycoplasma in a group of pigs.

The usefulness of the nested PCR for mycoplasma is also limited at this time by the fact that it is not known what percentage of animals have to be positive in order to reach the critical mass for clinical signs to appear. In this study, the number of positive animals appeared to be related to appearance of clinical signs and later to seroconversion. However, a threshold of positive animals for clinical signs to occur could not be established, especially since the sample size was inadequate for properly measuring prevalence. It is also possible that the "critical mass" varies with the strain involved, bacterial load, management factors, other pathogens present, number and density of animals, susceptibility, medication, and vaccination, so that each farm has its own "threshold." There are studies suggesting that the presence of *M. hyopneumoniae* in a farm does not necessarily imply an outbreak of disease.[5,28] However, none of these studies has shown that *M. hyopneumoniae* can remain in large populations of intensively housed pigs for several years without producing evidence of respiratory disease.

Further studies, involving more animals per age group—including farms without disease—and quantitating the amount of bacterial load, could give more insight and information on this putative "critical mass" and the effect and significance of carrier animals on disease.

Implications

- The nested PCR is able to detect *M. hyopneumoniae* earlier in infection than serology can.
- Detecting *M. hyopneumoniae* from nasal swabs offers new and valuable information in helping interpret serum profiles, determining when animals get infected, and how and where *M. hyopneumoniae* is being transmitted.
- This information has the potential to allow for better timing in the application of medication, vaccination, or management strategies.

Acknowledgments

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