Early *Mycoplasma hyopneumoniae* infections in European suckling pigs in herds with respiratory problems: detection rate and risk factors

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ABSTRACT: The present study aimed at estimating the detection rate of *M. hyopneumoniae* in 3-week-old pigs in different European countries and to identify possible risk factors. Nasal swabs from suckling pigs in 52 farms were collected for nested PCR analysis. Potential risk factors for respiratory disease were analysed with a multivariable logistic regression model. The average percentage of positive piglets was 10.7% (95% confidence interval, CI 7.4–14.2); at least one pig tested positive in 68% of herds. In 32% of the herds, more than 10% of piglets tested positive. Herds that vaccinated sows against swine influenza virus (SIV) had a significantly higher risk of a piglet being positive for *M. hyopneumoniae* (OR 3.12; 95% CI 1.43–6.83). The higher risk in case of SIV vaccination is difficult to explain, but may be due to the fact that pig herds with respiratory symptoms are more likely to be vaccinated against SIV, overlooking the possible influence of other respiratory pathogens such as *M. hyopneumoniae*. The present findings show that *M. hyopneumoniae* is widespread in 3-week-old piglets across different European countries.

Keywords: *Mycoplasma hyopneumoniae;* detection rate; risk factors; nasal swabs; suckling pigs

Infections with *Mycoplasma hyopneumoniae* occur worldwide and cause major economic losses to the pig industry. Control of *M. hyopneumoniae* can be accomplished by optimising management and housing practices, by antimicrobial medication and by vaccination. In many European countries, more than half of pig herds are vaccinated with the aim of controling infection (Maes et al., 2008). Different vaccination protocols are used, but in most instances the piglets are vaccinated once or twice in the farrowing unit or at weaning.

The spread of *M. hyopneumoniae* occurs by either vertical transmission from the sow to the offspring (Sibila et al., 2007) or by horizontal transmission between pigs (Meyns et al., 2004; Fano et al., 2007). Sows transmit *M. hyopneumoniae* to their offspring mainly by direct nose-to-nose contact, as there is no transmission via the intra-uterine route, nor by colostrum or milk. The importance of the sow for infection of the piglets is not clear and has been investigated only in a few studies including a limited number of pig herds. Goodwin et al. (1965) reported that younger sows are more likely to transmit *M. hyopneumoniae* to their piglets than older sows. Older sows, especially these in endemically infected herds, have a lower probability of harbouring *M. hyopneumoniae* in their respiratory tract (Clark et al., 1991). Calsamiglia and Pijoan (2000) found a higher percentage of young sows infected with *M. hyopneumoniae* in the upper respiratory tract, but the microorganism was also isolated from nasal samples taken from older sows (3rd to 7th parity).

Studies using nested PCR (nPCR) on nasal swabs from piglets before weaning in a limited number of herds have been performed. Calsamiglia and Pijoan (2000) tested animals in a three-site system and

found 7.7–9.6% positive pigs at 17 days of age. Ruiz et al. (2003) found 5.5–13.2% of piglets positive on a breeding farm at 19 days of age. Sibila et al. (2007) performed nasal sampling in piglets from one and three weeks of age; the percentage of positive piglets ranged between 0 and 6.4%. Much higher infection rates were obtained by Fano et al. (2007). They reported a prevalence of 2.5–51.8% in piglets at one day before weaning (17 days of age), in 11 batches of a multi-site production farm. One of the latest studies reports 2.0% prevalence in lung tissue from 201 suckling piglets from a highly populated pig area (Nathues et al., 2010). Although the presence of DNA in nasal swabs is not a 100% guarantee for infection, it is certainly an indication that these animals were exposed to the organism.

Sibila et al. (2007) performed nPCR on bronchial and tonsillar swabs of 37 pigs at three weeks of age and they showed the presence of mycoplasmal DNA in the tonsils of one pig and in the bronchial swab of two other pigs. Infection of piglets during the suckling period is particularly important, since piglets are transferred to other pens and are usually regrouped with other piglets at weaning. In this way, infected piglets may easily transmit the infection to other non-infected pigs during nursery.

To implement appropriate control measures and to further optimise the vaccination strategies, it is important to have a precise notion of how widespread this agent is in young pigs from different pig herds in different countries. Information regarding early infection is particularly important for the European pig industry, as early vaccination is widely practised (Maes et al., 2003) and farrow-to-finish pig herds constitute a major part of the industry. Apart from assessing the infection rate, it is also important to identify possible herd factors that may predispose to early infections in pigs. The aim of the present study was to estimate the detection rate of *M. hyopneumoniae* infections in piglets at three weeks of age in different European countries by analysing nasal swabs using nPCR. In addition, possible risk factors associated with *M. hyopneumoniae* infection in young piglets were investigated.

MATERIAL AND METHODS

Study herds

The study was conducted from May 2008 to March 2009 in nine European countries (Belgium, Denmark, France, Germany, Hungary, Italy, Poland, Spain and The Netherlands). The target population consisted of 52 pig herds. In each country, six single-site farrow-to-finish or sow herds were included, except for Germany, where four herds were included. The herds had to comply with specific selection criteria, such as a minimum herd size of 100 sows, presence of clinical respiratory problems related to *M. hyopneumoniae* (coughing in grower-finishing pigs) and no use of antimicrobials active against *M. hyopneumoniae* in piglets less than three weeks of age.

Nasal swabs

To obtain with 95% certainty a detection rate of at least 10% in the selected countries, a minimum of 30 piglets had to be sampled per herd. Hence, 30 nasal swabs were collected in each herd from piglets of 21 ± 3 days of age. The piglets within each herd were selected randomly from as many different sows as possible. In case there were less than 30 mother sows, two piglets per sow were selected.

Nasal swabs were obtained by deeply swabbing the nasal mucosa of one nostril using a cotton-tipped swab. The samples from each country were stored at –70°C.

Analysis of nasal samples

The material collected in each nasal swab was suspended in 200 µl phosphate buffered saline (PBS) and DNA was extracted using the Qiagen, DNAeasy Blood & Tissue Kit). To detect *M. hyopneumoniae* DNA, a nPCR protocol was used (Villarreal et al., 2009). The nested PCR reactions were performed using *Taq* polymerase. In the first step, a final volume of 20 µl contained 2 µl 10× PCR buffer, 2 µl 3mM $MgCl_2$, 0.36 µl 170µM dNTPs, 12.5 µl of high performance liquid chromatography water (HPLC-H_2O), 0.1 µl 0.03 IU/µl *Taq* polymerase (Gibco Invitrogen), 0.5 µl of each primer MHP950-1L (5'-AGGAACACCATCGCGATTTTTA-3') and MHP950-1R (5'-ATAAAAATGGCATTCCTTTTCA-3') and 2 µl 10-fold-diluted DNA solution.

In the second step, a final volume of 15 µl contained 1.5 µl 10× PCR buffer, 0.9 µl 3mM $MgCl_2$, 0.27 µl 170µM dNTPs, 10 µl HPLC-H_2O, 0.08 µl 0.03 IU/µl *Taq* polymerase, 0.5 µl primers MHP950-

2L (5'-CCCTTTGTCTTAATTTTTGCAA-3') and MHP950-2R (5'-GCCGATTCTAGTAC-CCTAATCC-3') and 1.5 µl 10-fold-diluted DNA solution.

The following PCR programme was used for both steps: initial denaturation at 94°C for 2 min; denaturation at 94°C for 30 s, annealing at 51°C for 30 s and extension at 72°C for 1 min; 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 1 min and extension at 72°C for 1 min; final extension at 72°C for 5 min, followed by cooling to 10°C. Nested PCR products were analysed by electrophoresis on 1.5% agarose gels in Tris-Borate-EDTA (TBE) buffer and visualised under ultraviolet illumination after staining with GelRed (Biotium). Positive and negative controls were used for both DNA extraction and amplification.

Herd data collection

A questionnaire related to respiratory disease and herd management practices was completed during a face-to-face interview with the farmer and when inspecting the sheds for each sampled herd. Thirty eight questions were included in the analysis. To ensure standardisation of the answers, most of these questions were closed (28) or semi-closed (10). The questionnaire was pretested in three pig herds before the start of the study. The questions were divided into six categories such as general herd information, sow population, purchase policy, housing conditions, feeding management and health control strategies. For logistical and language reasons, the completion of the questionnaires and the sampling were performed by the herd veterinarians in each country.

Statistical analyses

The detection rate in each herd was based on the number of positive nasal swabs detected by nPCR. In order to determine the risk factors associated with the detection rate of *M. hyopneumoniae* at herd level, the PROC GLIMMIX procedure (SAS 9.2, 2008) was used to fit a generalised mixed model for the presence of *M. hyopneumoniae* with country and herd nested in country as random effects and binomially distributed error term. The predictor variables with $P \leq 0.10$ in the univariate models were subsequently included in the multivariable analysis. A backward likelihood ratio selection criterion was used for the multivariable regression model in order to choose the variables that were significantly associated with the outcome variable ($P \leq 0.05$) at the multivariable level. At each step, a variable with the highest P-value (and > 0.05) was excluded from the model until no additional variables could be removed. One-way biologically plausible interactions between the independent variables were tested in the final model. The goodness-of-fit was evaluated by the ratio of the χ^2 statistic and the corresponding degrees of freedom with a value close to 1. Results were summarised using odd ratios with their 95% confidence intervals.

RESULTS

In total, 52 herds and 1 555 piglets were sampled for the presence of *M. hyopneumoniae*. Of all herds included in the study, 28.6% were farrow-to-finish herds, 26.5% and 24.5% were sow herds from which the piglets were moved to another farm after weaning or at 20–25 kg, respectively, and the remainder of the herds (20.4%) raised a part of the fattening pigs on the same site and partly moved pigs after weaning or at 10 weeks of age. The sows from the sampled pigs had following parity distribution: 1st to 2nd parity: 39%; 3rd to 4th parity: 30%; 5th to 7th parity: 27%; and >7th parity: 4%. Thirty-three percent of the herds vaccinated sows against *M. hyopneumoniae*.

The total average percentage of positive pigs within the herds was 10.7% (95% CI 7.4–14.2). In herds vaccinating against *M. hyopneumoniae* 8.4% (95% CI 7.6–9.1) were infected, whereas only 2.6%

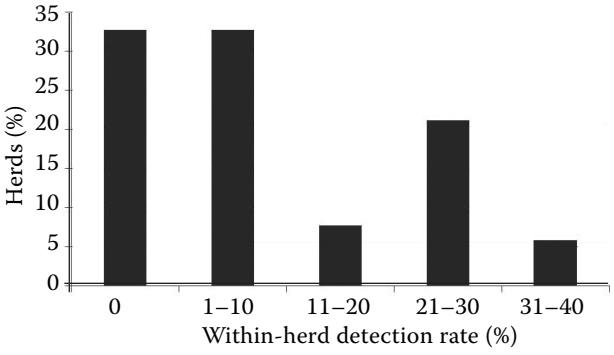

Figure 1. Distribution of herds according to within-herd detection rate of *M. hyopneumoniae* in 3-week old piglets. Fifty-two pig herds from different European countries were included; 30 piglets per herd were sampled

Table 1. Detection rate of *M. hyopneumoniae* in nasal swabs from 3-week old pigs in different EU countries

Country	Number of positive herds/ Number of herds sampled	Number (%) of positive piglets[a]	Minimum–maximum % of positive pigs
Belgium	4/6	6 (3.3)	0.0–10.0
Denmark	3/6	3 (1.7)	0.0–3.30
France	6/6	38 (21.1)	10.0–30.0
Germany[b]	3/4	23 (18.7)	0.0–26.7
Hungary	3/6	18 (10.0)	0.0–30.0
Italy	5/6	40 (22.1)	0.0–36.7
Poland	4/6	17 (9.7)	0.0–26.7
Spain	2/6	9 (5.0)	0.0–20.0
The Netherlands	5/6	14 (7.7)	0.0–16.1
Total (%)	35/52 (67.3)	168/1555 (10.7)	0.0–36.7

[a]fifty-two herds were sampled; 30 piglets were sampled in each herd: 6 herds per country, 30 pigs per herd = 180 pigs per country (only 4 herds or 120 pigs in Germany); Only 25 samples were taken from one herd in Poland
[b]only 4 herds were sampled

(95% CI 1.8–3.4) were infected in the herds not vaccinating against *M. hyopneumoniae*. Twenty eight percent of the herds practiced sow vaccination against *M. hyopneumoniae* before their first gestation, accounting for 3.1% of the *M. hyopneumoniae* positive piglets. No significant difference was found between these and the herds not practicing sow vaccination ($P > 0.05$).

The minimum within-herd detection rate was 0% and the maximum within-herd detection rate was 37% (Figure 1). The percentage of herds with at least one positive tested pig was 68% (95% CI 51.7–83.4).

The number of positive herds and positive animals in the different countries are presented in Table 1. France, The Netherlands, Italy and Germany were the countries with the highest number of herds positive for *M. hyopneumoniae*. In France, 100% of the herds were positive. The highest within-herd detection rates were found in Italy, France and Germany.

Three of 38 variables were significant in the univariate analysis ($P \leq 0.10$), namely sow vaccination against swine influenza (SIV), sow vaccination against atrophic rhinitis and the presence of a slaughterhouse within a distance of 5 km from the herd (Table 2). In total, 27% and 28% of herds vaccinated their sows against SIV and atrophic rhinitis, respectively. In 10% of the herds, a slaughterhouse was located within 5 km of the herd.

These three variables were subsequently used in the multivariable model. As presented in Table 3, only sow vaccination against SIV was significantly ($P < 0.05$) and positively associated with the preva-

Table 2. Potential risk factors ($P < 0.10$) associated at herd level with the detection rate of *M. hyopneumoniae* in piglets of three weeks of age after univariate analysis

Variables	Number of herds	Level	Number (%) of herds per level	Mean % of positive herds per level	*P*-value
Sow vaccination against swine influenza	52	no yes	38 (73.1) 14 (26.9)	5.0 15.5	0.014
Sow vaccination against atrophic rhinitis	52	no yes	37 (71.2) 15 (28.2)	5.4 13.4	0.029
Presence of slaughterhouse within 5 km from pig herd	49	no yes	44 (89.8) 5 (10.2)	6.6 21.6	0.034

Table 3. Risk factors ($P < 0.05$) for *M. hyopneumoniae* positivity in 3-week-old piglets in nine European countries in the final multivariable logistic regression model[a]

Variable	Level	β-coefficient	Odds ratio (95% CI)	*P*-value
Sow vaccination against swine influenza	no		1	0.023
	yes	1.14	3.12 (1.43–6.83)	
Sow vaccination against atrophic rhinitis[b]	no		1	0.052
	yes	0.91	2.48 (0.86–5.45)	

[a]generalized χ^2; degrees of freedom 0.95
[b]variable was included in the final model, since the *P*-value was borderline not significant

lence of *M. hyopneumoniae* in the piglets (OR 3.12; 95% CI 1.43–6.83) when adjusted for the other covariates. Vaccination against atrophic rhinitis was not significantly associated with the prevalence of *M. hyopneumoniae* in piglets, but the factor was retained in the final model because the *P*-value was borderline not significant ($P = 0.052$). The goodness of fit statistic was 0.95, close to and not differing significantly from 1, demonstrating that this model describes the data well.

DISCUSSION

The present study assessed the detection rate of *M. hyopneumoniae* in piglets of three weeks of age in different types of pig herds located in different European countries. The results showed that there was evidence of *M. hyopneumoniae* infection in 3-week old piglets in two thirds of the studied herds. In most of the positive herds, the detection rate was between 1 and 10%. In some herds, more than 30% of the piglets were positive. This suggests that, in the selected herds, early infections with *M. hyopneumoniae* are important (Sibila et al., 2004, 2007; Fano et al., 2005). As specific criteria were used to select the herds (clinical problems in grower-finishing pigs attributed to this pathogen), the results may not be generalised to all pig herds within the EU, but only to herds meeting the selection criteria. However, in terms of management and housing conditions, the selected herds can be considered to be representative for many other pig herds in the EU (Sibila et al., 2004; Beilage et al., 2009).

Nested PCR on nasal swabs was used to analyse the samples. This diagnostic tool is suitable for detection of *M. hyopneumoniae* under field conditions in suckling pigs and nursery pigs (Otagiri et al., 2005; Sibila et al., 2007), and can detect 5.1×10^6 ng *M. hyopneumoniae* DNA or as few as four organisms/µl reaction mixture (Gebruers et al., 2008). To avoid false positive results due to contamination in the nPCR, appropriate positive and negative controls were used during DNA extraction and amplification. The results should be interpreted at the herd level, not at the individual level (Otagiri et al., 2005).

M. hyopneumoniae attaches to and multiplies on the ciliated epithelium of the trachea, bronchi and bronchioles (Zielinski et al., 1993). Consequently, the nose is not the primary site of multiplication of *M. hyopneumoniae* organisms. It is therefore likely that the present results underestimate the number of infected animals in the selected herds and that a higher detection in recently weaned piglets would have been obtained from bronchoalveolar lavage fluid (BALF) (Moorkamp et al., 2009). Taking BAL fluid is possible under field conditions, but is less practical and more time-consuming than taking nasal swabs.

Serology would not work in the present study as it is not possible to discriminate between serum antibodies following infection and maternally derived antibodies. In addition, it takes at least 4–8 weeks before contact-infected pigs may show detectable serum antibodies (Feld et al., 1992). Isolation of the bacterium is the gold standard for diagnosis of *M. hyopneumoniae*, but for the present study this method is inappropriate at the herd level. Lung tissue or BAL fluid should be used instead of nasal swabs; however, the taking of these samples is very expensive, time-consuming and special medium is required.

Although sow vaccination against *M. hyopneumoniae* is not commonly practiced, twenty eight percent of the sows in our study had been vaccinated before their first gestation. The detection rate of *M. hyopneumoniae* in the piglets was not significantly different between herds practicing sow vaccination

and those not practicing vaccination. One could expect that vaccination in sows decreases the number of *M. hyopneumoniae* organisms shed by the sow and increases the level of maternal antibodies provided to the offspring. These factors could lead to a slight decrease in the number of PCR-positive pigs at weaning (Sibila et al., 2007). The absence of a difference in the present study can be due to differences in vaccination strategies employed by the herds (e.g., timing of vaccination, age of the sows at vaccination, type of vaccine) and the large variability in serological response (30–100%) that may occur following sow vaccination (Thacker et al., 1998).

Herds vaccinating sows against SIV had a three times higher risk for having test-positive pigs compared to herds not vaccinating against SIV. From a biological point of view, it is not only unlikely, but also hard to explain how SIV vaccination would predispose to early *M. hyopneumoniae* infections. The significant and positive association may be due to the fact that herds with respiratory problems are more likely to vaccinate their sows against SIV. As most of the respiratory problems are due to multiple infections and also to non-infectious factors (Thacker et al., 2002), it is possible that in these herds, *M. hyopneumoniae* and/or other pathogens are involved in respiratory problems in addition to SIV.

These results indicate that before a vaccination program is implemented, it is imperative to have a full picture of the problem in a herd and not to rely solely on the evaluation of the clinical symptoms. Performance of additional laboratory examinations (e.g., postmortem examination of dead animals, serology of different age groups) is required to investigate the different pathogens involved in the problem before establishing an aetiological diagnosis. Otherwise, vaccination programs may not be sufficient to control the main pathogen(s) in the herd.

Finally, some comments should be made with regard to the use of a cross-sectional study design and the causality of the risk factor. Only one group of piglets from each herd was sampled. Cross-sectional studies measure events at a particular time and therefore cause and effect are sometimes difficult to separate. The risk factor found in this study did not fulfil the criteria used to transpose observed associations into a causal relationship (Susser, 1986). As already stated and explained, it was likely due to confounding (Thrusfield, 1997). Given that only one group of piglets was investigated within each herd, a sufficient number of piglets was tested and also a large number of herds from different European countries were included. This increases the validity of the results to other pig herds complying with the selection criteria.

This study showed that using nested PCR on nasal swabs, *M. hyopneumoniae* infections in 3-weeks old pigs occur commonly in European pig herds suffering from respiratory disease in grow-finishing pigs. No significant risk factors directly related to *M. hyopneumoniae* infections were found, but the significant variation in within-herd prevalence between herds indicates the need for further studies aimed at identifying risk factors involved in the transmission of *M. hyopneumoniae* in suckling pigs. The results also reinforce the need of establishing a precise and detailed diagnosis before implementing a vaccination scheme, as different pathogens may be involved in respiratory disease.

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