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CHRONOLOGICAL STUDY OF *MYCOPLASMA HYOPNEUMONIAE*
INFECTION, SEROCONVERSION AND ASSOCIATED LUNG LESIONS IN
VACCINATED AND NON-VACCINATED PIGS

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Abstract:

A field trial was conducted to study *Mycoplasma hyopneumoniae* (Mh) infection dynamics by nested polymerase chain reaction (nPCR) and serology in pigs of a farm affected by enzootic pneumonia (EP). Moreover, correlation of Mh detection at different respiratory tract sites with presence of EP gross and microscopic lung lesions was assessed. These parameters were studied and compared between vaccinated (two doses at 1 and 3 weeks of age versus one dose at 6 weeks of age) and non-vaccinated pigs. Animals were monitored from birth to slaughter by nPCR from nasal swabs and by serology. From 3 to 22 weeks of age, an average of 3 pigs per treatment and per batch were necropsied (n=302). The remaining pigs were sent to the slaughter (n=103). Nasal, bronchial and tonsillar swabs were taken from the necropsied/slaughtered pigs; gross and microscopic EP-suggestive lung lesions were also assessed. Single and double vaccination resulted in earlier seroconversion and higher percentage of Mh seropositive pigs compared to control group. At slaughter, double vaccinated pigs showed lower percentage of EP-compatible gross lung lesions and lower *Mh* prevalence at upper respiratory tract sites (nasal cavity and tonsil) than control pigs.

Keywords: *Mycoplasma hyopneumoniae*; infection; lesions; serology; vaccination

1. Introduction

Mycoplasma hyopneumoniae (Mh) is the etiologic agent of enzootic pneumonia (EP) in pigs, a chronic respiratory disease that causes major economic losses to the pig industry (Thacker, 2006). Detrimental effects of EP are mainly associated to treatment costs, reduced performance in growing and finishing pigs and the consequent lower market price of the carcass. The decrease in average daily gain and the lower carcass weight is correlated with the increase of pneumonic lung lesions (Pommier et al., 2000).

Vaccination with commercial bacterins is one of the tools used to ameliorate the effects of Mh infection. Major advantages of Mh vaccination include improvement of production parameters, shorter time to reach slaughter weight, reduction of clinical signs and lung lesions and, occasionally, lower mortality and improvement of carcass quality (Haesebrouck et al., 2004). Traditional Mh vaccination protocols include two doses of bacterin, although the use of one shot vaccines is nowadays commercialized as well (Baccaro et al., 2005). Vaccination with a single-dose bacterin dose has the main advantage of reducing labour and causing less stress to the animals (Baccaro et al., 2005; Dawson et al., 2002).

The efficacy of one or two doses of Mh vaccination varies depending on the commercial vaccine product, schedule, farm type and the study design used (Andreasen et al., 2001; Dawson et al., 2002; Kyriakis et al., 2001; Pallares et al., 2000; Ruiz et al., 2002). Most of published field studies have measured the effect of vaccination against Mh on lung lesion reduction and improvement of production parameters, but few of them have addressed Mh infection dynamics (Baccaro et al., 2005; Martelli et al., 2006; Ruiz et al., 2002).

Therefore, the objectives of the present work were: 1) to study Mh infection dynamics by nPCR and serology in pigs of a farm affected by EP, and 2) to correlate

Mh detection at different respiratory sites with presence of EP gross and microscopic lung lesions in the same farm. These parameters were studied and compared in pigs vaccinated with two and one dose of a commercial bacterin and with non-vaccinated pigs.

2. Materials and methods

2.1 Farm

A one site farrow-to-finish herd with 240 sows and continuous flow production system (in nursery and finishing units) was selected. The farm had respiratory clinical signs in fattening pigs (coughing) and EP-compatible lung lesions at slaughter. To confirm Mh-related disease, a nPCR from nasal swabs and evaluation of gross lung lesions from 34 slaughtered pigs were performed. Moreover, blood samples from other 21 slaughtered pigs were also taken. All 34 (100%) tested pigs were positive to Mh by nPCR and had a mean macroscopic lung score of 9.16 based on the classification by Hannan et al. (Hannan et al., 1982). On the other hand, 13 out of 21 (62%) were seropositive to Mh.

2.2 Study design

This was a blinded, randomized and controlled field trial. A total of 542 pigs (268 female and 274 male) from 69 different sows of 6 different farrowing batches were included in this study. The parity range of selected sows was from 1 (n=5) to 8 (n=2), with a mean of 4.4 parity number. No Mh vaccination or medication protocols were being performed before the start of the study. Piglets were ear-tagged at 1 week of age and randomly distributed in the following treatment groups: 1) Two dose group (n=182): pigs that received 2 ml of vaccine at 1 and 3 weeks of age and 2 ml of

phosphate buffer saline (PBS) at 6 weeks of age; 2) One dose group (n=176): pigs injected with 2 ml of PBS at 1 and 3 weeks of age and 2 ml of vaccine at 6 weeks of age; 3) Control group (n=184): pigs that received 2 ml of PBS at 1, 3 and 6 weeks of age. In order to distribute animals to different treatment groups, piglets from one sow were assigned correlatively to the 3 treatments: first randomly selected piglet was assigned to “two doses of vaccine” treatment, second piglet to “one dose of vaccine” treatment and the third one to the control group. This criterion was followed until each piglet from the same sow was assigned to one out of 3 treatments. Afterwards piglets from the next sow were distributed continuing the treatment order from the previous sow. All vaccinations, as well as injections of PBS, were administered intramuscularly according to the recommendations of the manufacturer. The vaccine product used was a commercial batch (lot 5JZ2) of MYPRAVAC SUIS® (Hipra [Girona] Spain), which is a suspension of inactivated Mh strain J with a carbomer/levamisol adjuvant. Using this treatment design, all piglets received the same number of injections. To minimize the potential litter effect on Mh vaccination, each litter had 2 to 3 pigs of each treatment. To avoid deviations due to management or facilities conditions, a similar number of animals of each treatment group were included in all pens.

Piglets were all housed in similar conditions, received the same feed and were subjected to the same management practices (castration, iron injection, teeth clipping and tail docking). Animals were weaned at 17 to 23 days of age and transferred to the nursery units, where litters from different sows were mixed. At 9 weeks of age, pigs were moved to the growing-finishing units and they were distributed taking into account the body condition, following the farmer criteria. All three treatments were represented in each pen in both, nursery and growing-finishing barns. Treatments, housing,

husbandry and slaughtering conditions conformed to the European Union Guidelines and Good Clinical Practices.

2.3 Sample collection

Nasal swabs (Culturette TM, Becton Dickinson, Sparks, USA) and blood samples (5 ml Venoject, Terumo Europe, Madrid, Spain) from all animals were taken at 1, 3, 6, 9, 12, 15, 18, 22 and 25 weeks of age. Nasal swabs were obtained by swabbing the nasal mucosa of both nostrils reaching deeply into turbinates. Moreover, blood sample from sows was collected at 1 week post-farrowing (the same day as piglet sampling at 1 weeks of age).

The initial number of pigs included in the study (n=542) decreased over time because, from 3 weeks of age onwards, necropsies of randomly selected pigs of each treatment were performed. To select these animals, a random table was done taking into account that animals from different treatments and sows were represented in each necropsy day. Thus, an average of 3 pigs per treatment and batch were necropsied at 3 (n=39), 6 (n=36), 9 (n=37), 12 (n=37), 15 (n= 48), 18 (n=51) and 22 (n=54) weeks of age (total of 302 pigs). Necropsied pigs were euthanized with an intravenous overdose of sodium pentobarbital. Pigs that reached the market weight were slaughtered at 25–28 weeks of age (n=103). From the 542 animals initially included in the study, 50 (9.2%) nursery piglets died due to central nervous clinical signs probably associated to *Streptococcus suis* infection. On the other hand, 71 (13%) growing and finishing pigs died due to mainly respiratory and wasting problems. Specific diagnosis of each dead pig was not recorded since the farm was visited only the sampling days. Therefore, performing the necropsy of death animals was not possible. Moreover, there were 16

finishing pigs that lost the ear-tag at slaughter. From that 16 (3%) pigs data recorded arrived until 22 weeks of age.

From the necropsied (n=02) and slaughtered (n=102) pigs, tonsillar and bronchial swabs (Culturette TM, Becton Dickinson, Sparks, USA) and lung tissue were taken. Tonsillar swabs were obtained by swabbing the surface of the tonsil thoroughly but gently. Bronchial swabs and lung samples were obtained from the lungs already separated from the carcass; samples were taken from cranial and middle lobes and from pneumonic areas when present. Afterwards, these tissue pieces were fixed by immersion in 10% buffered formalin. All collected samples were individually identified and transported (swabs and blood samples in refrigeration) to the laboratory, where they were stored at -80°C until their processing.

2.4 DNA extraction and nPCR to detect Mh nucleic acid.

Nasal, tonsillar and bronchial swabs were resuspended in 1 ml of sterile PBS and vigorously vortexed. Four hundred microliters of the suspension were used for DNA extraction. DNA was extracted using a commercial kit (Nucleospin® Blood, Macherey-Nagel GmbH & Co KG Düren, Germany) and samples were processed according to manufacturer's instructions. To test for potential contaminations during the extraction procedure, a negative control was included using PBS as extraction substrate in each group of extracted samples.

A nPCR to detect Mh was performed using previously described primers and conditions (Calsamiglia et al., 1999). To minimize the risk of contaminations, each stage of the nPCR process (DNA extraction, DNA amplification and electrophoresis) was carried out in three separated rooms. In each nPCR reaction, a negative control (consisting of millipore water) every four test samples as well as a positive control

(DNA extracted from a pure culture of Mh) in the last position were included. The amplified products (352 bp) were run in a 2% agarose gel with 0.05 mg/ml of ethidium bromide, and then visualized and pictured (Bio-1d V.96, Vilber Lourmat, France).

2.5 ELISA technique to detect Mh antibodies

Blood samples were centrifuged at 760 g during 10 minutes at 4°C and the sera obtained were frozen at -80°C until used. Presence of antibodies to Mh was tested by a monoclonal blocking ELISA (CIVTEST SUIS® MYCOPLASMA HYOPNEUMONIAE, HIPRA [Girona], Spain). The sensibility and specificity of the test used were, 93% and 96% respectively (Sorensen et al., 1992). The inhibition percentage (IP) was calculated taking into account the optical densities (OD) of each sample as well as the one of the negative control (negative value). ELISA results interpretation, based on IP values, was as follows: IP < 50%, negative; IP > 50%, positive. This percentage was obtained from the following formula:

$$IP (\%) = \frac{\text{Mean negative value OD} - \text{sample OD}}{\text{Mean negative value OD}} \times 100$$

In addition, sera from slaughtered pigs (n=102, serum from one animal was lacking) were tested for antibodies to: porcine respiratory and reproductive syndrome virus (PRRSV) (indirect ELISA, CIVTEST SUIS PRRS® European variant, Hipra; interpretation: positive >20); H1N1, H3N2 or H1N2 subtypes of swine influenza virus (SIV) (indirect ELISA, CIVTEST SUIS Influenza®, Hipra; interpretation: positive >20); Aujeszky's disease virus (ADV) (blocking ELISA, CIVTEST SUIS ADV®, Hipra, interpretation: positive >90); and *Actinobacillus pleuropneumoniae* (indirect ELISA, CIVTEST SUIS App®, Hipra, interpretation: positive >20).

2.6 Pathological studies

Extension of EP-compatible gross lesions (craneo-ventral pulmonary consolidation) was assessed taking into account the localization of the lesions and the relative volume of each lobe (Hannan et al., 1982). EP-compatible macroscopic lung lesions were registered on a lungs' diagram and given from 1 to 5 points per lobe depending on the extension of these lesions. The maximum total score possible per lung was 35. Once given the lesional scoring per lobe, the scoring was normalized taking into account the relative volume of each lobe (Hannan et al., 1982).

After fixation for 24 h in 10% buffered formalin, lung pieces were dehydrated, paraffin embedded, sectioned at 4 μ m and stained with haematoxylin and eosin for histological examination using a light microscope. Histopathologic lesions were graded from 0 to 4, following previously described criteria (Calsamiglia et al., 2000). Absence of lesions scored 0. Lesions characterized by inflammation non suggestive of EP were scored 1 and 2; scores 3 and 4 were considered suggestive of Mh infection. Score 3 consisted of lesions with perivascular and peribronchiolar lymphoplasmacytic hyperplasia, pneumocyte type II hyperplasia and edema fluid in the alveolar spaces with neutrophils, macrophages and plasma cells. Score 4 was considered when lesions with the characteristics of score 3 together with the presence of evident peribronchial and perivascular lymphoid follicles. Lung tissue scoring was performed in a blinded fashion. In the statistical analysis this variable was categorized in two groups: presence of non-suggestive (0, 1 or 2 histopathologic scores) and suggestive (3 or 4 histopathologic scores) EP microscopic lung lesions.

2.7 Statistical analyses

Bivariate analyses using contingency tables (Chi-square statistics or Fisher's exact test for 2x2 tables) were used to compare following parameters among different treatments groups: 1) number of seropositive sows 2) nPCR Mh detection at nasal swabs at different sampling weeks; 3) nPCR Mh detection in three respiratory sites at necropsy or slaughter age; 4) Mh antibodies detection by ELISA at different sampling weeks; and 5) microscopic lung lesion scoring globally (including all ages studied within a group) and by necropsy age.

Non-parametric statistical analysis (Kruskal-Wallis statistic) was used for testing differences in gross lung lesion scoring among animals from different treatments groups. On the other hand, Wilcoxon statistics were used to assess the relationship between: 1) gross lung lesion scoring and detection of the pathogen in the three different sampling sites in the necropsied (n=302) and slaughtered (n=103) pigs; and 2) gross lung lesion scoring at slaughter (n=103) with nPCR results in nasal cavities at different sampling times (3, 6, 9, 12, 15, 18, and 22 weeks of age).

Statistical analyses were performed with the SAS system for windows version 8.0 (SAS Institute Inc, Cary, North Carolina, USA). Statistical significance level was set at $\alpha=0.05$.

The vaccine efficacy in reducing lung score was calculated, using group median lung scores (Jones et al., 2005), as follows: Vaccine efficacy = [(median non-vaccinated group score – median vaccinated group score)/ median non-vaccinated group score] x 100.

3. Results

From the 69 sows included in this study, 20 (29%) were seropositive at 1 week post-farrowing. No statistical significant differences between numbers of seropositive

sows neither per treatment nor per parity number were found. On the contrary, sow parity number was significantly associated with serologic status of piglets at 1 one week of age ($p=0.006$). At that age, all piglets ($n=35$) coming from gilts ($n=5$) were *Mh* seronegative. Nevertheless, it should be taken into account that the five gilts included in this study were seronegative. On the other hand, sow parity number was also significantly related with detection of *Mh* in their piglets by PCR at 9 ($p=0.003$) and 12 ($p=0.0004$) weeks of age. Specifically, was detected in higher percentages in piglets coming from gilts (5 out of 24 [20.8%] and 12 out 19 [63.1%] at 9 and 12 weeks of age respectively) than from sows of intermediate (12 out of 263 [4.5%] and 69 out 220 [31.3%], at 9 and 12 weeks of age respectively) or high (11 out of 105 [10.4%] and 18 out 94 [19.1%], at 9 and 12 weeks of age respectively) parity number.

Globally, *Mh* nPCR detection percentages at nasal cavities were very similar in all three treatment groups (Table 1). From 1 to 9 weeks of age, *Mh* was detected in nasal cavities in relatively low proportion ($<10\%$) in all groups. On the contrary, *Mh* detection percentages tended to increase in all three treatments groups from 12 weeks of age until the slaughter age. At 6 weeks of age, there were higher percentages of pigs positive by nPCR in one dose group than the control group of pigs ($p < 0.05$). Moreover, at slaughter age, percentage of nPCR positive pigs at nasal cavities was higher in the group of animals that received two doses of vaccine than in control pigs ($p < 0.05$).

Mh serological results from all tested pigs are summarized in Figure 1. A similar percentage of seropositive pigs was observed in all treatments groups at 1 and 3 weeks of age. After the decrease of this initial percentage, an earlier seroconversion to *Mh* was observed in vaccinated groups compared with non-vaccinated pigs. Significant differences between treatments regarding the percentage of *Mh* seropositive pigs were

observed at 6 (two dose versus one dose and control groups), 9 (two dose versus control groups) and from 12 to 22 (both vaccinated groups versus control groups) weeks of age. At slaughter age, percentages of seropositive pigs to Mh were not different between treatment groups.

From 542 pigs included in this study, 405 (135 for the two doses treatment, 133 for the one dose treatment, and 137 for the control group control treatment) were necropsied/slaughtered at different sampling times. From these 405 animals, 4 nasal swabs, 4 bronchial swabs, and 4 tonsillar swabs were missing.

Overall, Mh was detected in greater percentages in bronchial (227/401, 56.1%) followed by tonsillar (136/401, 33.9%) and nasal swabs (122/401, 30.4%). Moreover, detection of Mh at bronchus by nPCR had a relationship with detection at nasal cavity ($p<0.05$) and tonsil ($p<0.05$) swabs; detection of Mh DNA at tonsillar had also a relationship with nasal swabs ($p<0.05$). Results of nPCR detection at nasal cavity, bronchus and tonsil of necropsied pigs at different ages are summarized in Tables 2, 3 and 4. Mh detection at nasal, bronchial and tonsillar swabs from necropsied/slaughtered pigs was lower than 10% in all treatments groups from 1 to 9 weeks of age. Percentage of bronchial nPCR positive pigs increased by 12 weeks of age, and it was maintained high until slaughter age where approximately an 80% of the pigs were positive by nPCR. On the contrary, Mh detection in nasal cavity and tonsil showed a soft peak at 15 weeks of age in all studied groups, followed by an irregular profile. At slaughter age, control group had greater percentage of Mh detection at nasal cavity and tonsil than pigs vaccinated with two doses of bacterin ($p=0.0128$ and $p=0.0082$, respectively).

Pulmonary consolidation suggestive of EP was observed in a number of necropsied animals. From a total of 404 pigs, 124 (30.6%) had no macroscopic lung lesions, 221 (54.7%) had less than 25% (8.75 out of 35 scoring points) affected lung

affected, 36 (8.9%) showed between 25 to 50% (17.5 out of 35 scoring points) damaged lung, 18 (4.5%) with 50 to 75% (26.25 out of 35 scoring points) lung affected and, finally, 5 (1.2%) had more than 75% of the lung affected. No gross lung lesions compatible with EP were assessed in one pig due to a severe, diffuse fibrous pleuritis observed at the moment of necropsy. Although EP-compatible gross lung lesions (pulmonary craneo-ventral consolidation) were observed in animals of different ages and treatments, the extension of these lesions increased at 12-15 weeks of age onwards (Table 5). At 25 weeks of age, median lesional scores were lower in two-dose vaccinated pigs (score value of 2.18) and one-dose vaccinated animals (score value of 2.42) compared to control pigs (score value of 5.70); however, only significant differences ($p=0.05$) were confirmed between the two dose and the control groups. Vaccine efficacy in reducing lung lesions score was 61.8% and 57.8% when comparing two and one dose vaccination groups, respectively, with controls animals.

Overall, EP gross lung lesions were associated with prevalence of Mh in nasal cavity, tonsil and bronchus (Table 6) at the necropsy or slaughter age; median EP-compatible lung scores were higher ($p<0.05$) in pigs positive by nPCR than negative ones, independently of the treatment received. Moreover, in the animals sent to the slaughter ($n=103$), an association of the average lesional scores with nPCR detection in nasal swabs at 3 ($p=0.057$, Chi-square value 3.64) weeks of age was seen; in other words, animals with a positive nPCR nasal swab at 3 weeks of age ($n=7$) tend to have higher median EP-compatible lung lesions (5.0 ranging from 2.4 to 25) at slaughter age than pigs with a negative result ($n=96$, 2.7 ranging from 0 to 28).

From the 405 necropsied/slaughtered pigs, 63 (15.5%) did not have any microscopical lung lesions (score 0), 94 (23.2%) showed EP non-suggestive microscopic lung lesions (scores 1 or 2) and 248 (61.2%) showed EP suggestive microscopic lung

lesions (scores 3 or 4). Percentage of pigs showing these lesions increased with the age in all three treatments groups (Table 7). No statistically significant differences on EP suggestive microscopic lung lesions were observed between the studied groups in a given age.

Percentage of seropositive pigs at slaughter to PRRSV, ADV, SIV and *A. pleuropneumoniae* were 100, 90, 81 and 39%, respectively. No significant differences on percentages of seropositive pigs to these respiratory pathogens (data not shown) were detected among treatment groups. However, control pigs (n=35) showed a statistically significant higher ($p=0.02$) serologic values against PRRSV (164.6 ± 27.8) than pigs vaccinated with two (140.6 ± 48.8) and one dose (141.2 ± 40.8) of bacterin.

4. Discussion

The present study describes the pattern of Mh infection and seroconversion in vaccinated and non-vaccinated pigs in a farrow-to-finish operation with EP associated clinical signs. Monitoring pigs from birth to slaughter with sequential necropsies every 3 weeks allowed understanding the effect of different vaccination schedules on localization of Mh in different respiratory tract sites and EP lung lesion evolution.

Differences in EP-compatible lung lesion scores were observed at slaughter age between double vaccinated and control pigs, coinciding with other field trials in which the same (Llopart et al., 2002) or other (Kyriakis et al., 2001; Maes et al., 1999; Pommier et al., 2000) commercial two-dose vaccines were administered at weaning age. Traditionally, percentage of lung lesion reduction in vaccinated pigs compared to control pigs is one of the main parameters used to measure Mh vaccine efficacy (Jones et al., 2005). In the present study, vaccination at 1 and 3 weeks of age resulted in a reduction of EP-compatible gross lung lesions of nearly 62% when compared to the

controls pigs. Similar or lower lung lesion reduction has been also found in other field studies using two doses of Mh vaccine (Kyriakis et al., 2001; Maes et al., 1999; Scheidt et al., 1994). On the other hand, single vaccination at 6 weeks of age resulted in 57.8% lung lesion reduction; however, the extent of these lesions were not statistical significantly different from the control group, probably due to relatively low mean lung lesional score observed (4.17 versus 7.71, respectively). Obtained results for single Mh vaccination are in the same line than those achieved in other studies (Baccaro et al., 2005; Dawson et al., 2002) in which vaccination was performed at weaning age. On the contrary, two field studies in which one dose vaccine was administered at 10 weeks of age (Kyriakis et al., 2001; Pommier et al., 2000) found relatively low (<20%) lung lesion reduction, probably due to the short time between vaccine application and timing of infection. In the present study, single vaccination was administered approximately 6 to 9 weeks before most of the pigs had Mh at bronchus, suggesting that pigs had enough time to develop a protective immune response able to reduce the extent of gross lung lesions.

Increase of percentage of animals showing EP-compatible macroscopic lesions coincided with the increase of Mh detection at all three respiratory sites (12-15 weeks of age) but not with the increase of percentage of pigs showing EP-suggestive microscopic lung lesions, which occurred earlier (6-9 weeks of age). This coincidence on time between infection and macroscopic lung lesions but not with microscopic ones, suggest that these latter lesions might be caused by other pathogens different from Mh, such as SIV (Thacker et al., 2001). In fact, seroconversion against SIV was detected in slaughtered pigs of these groups, indicating that this virus had circulated in that unit. However, the detection of microscopic lesions before the observation of gross lesions would also suggest initial phases of Mh lung colonization

The fact that EP clinical signs (mainly coughing) at individual level in this study were not recorded, prevent us from knowing the number of animals showing clinical signs as well as the exact time of disease appearance and its relationship with lung lesions evolution.

The fact that the percentage of seropositive sows was not different among treatments, and that the percentage of seropositive pigs at 1 and 3 weeks of age was similar in all treatments, suggest that in the conditions of this study, there were no influence of sow serologic status on the response to the treatment of piglets.

The statistical association between gilt serology and seronegative pigs at 1 week of age is explained by the lack of seropositive gilts included in the study. On the other hand, the fact that all piglets from these gilts were seronegative and had greater probabilities of being infected by *Mh* at 6 and 9 weeks of age, may suggest a beneficial effect of maternal derived antibodies against *Mh* colonization.

Under field conditions, seroconversion to *Mh* is usually quite delayed and occurs mainly in growing and finishing units (Leon et al., 2001). In the present study, seroconversion was observed 3 weeks before in both vaccinated groups (between 9 to 12 weeks of age) than in control animals (between 12 to 15 weeks of age). Both vaccination groups had a very similar serumprofile, only differing statistically at 6 weeks of age. At that week, percentage of seropositive pigs in the two dose group did not decline to the low percentage as the one dose group did, indicating a humoral immune response due to vaccination. On the other hand, at 12 weeks of age, percentage of seropositive pigs from one dose group reached nearly the same percentage as the two dose group (21 and 23%, respectively). Therefore, obtained results indicated the capability of both vaccine schedules to elicit a detectable humoral immune response to *Mh* after vaccination.

Percentage of seropositive pigs increased progressively towards the end of the finishing period in all three groups, indicating that both vaccinated and non-vaccinated pigs produced also a humoral detectable response to natural infection. In this study, Mh was detected at upper respiratory tract levels (nasal cavity and tonsil) and at the site of pathogen's action, the bronchus (Thacker, 2006), confirming previous published results (Sibila et al., 2004). Bacterioprofiles obtained in the present work indicated that, although most pigs became infected in the fattening units, Mh early colonization did sporadically occur at upper and lower respiratory sites (Baccaro et al., 2005; Sibila et al., 2006).

Detection of the pathogen in all treatment groups and ages confirmed that Mh vaccination did not prevent from infection (Maes et al., 1999); however, vaccination was related with a lower Mh prevalence at slaughter age in upper respiratory airways (nasal cavities and tonsils), as previously described (Baccaro et al., 2005; Ruiz et al., 2002). This result is probably due to a reduction of Mh excretion (Baccaro et al., 2005). The fact that no differences in Mh prevalence at bronchus were observed may be related to the qualitative technique used to detect this pathogen. It is possible that vaccinated pigs may have lower Mh load at bronchus and, consequently, may shed less pathogen particles (detectable at the upper respiratory tract) than control pigs. A quantitative method for Mh detection would help to confirm if vaccination reduces bacterial load at bronchus, as previously found by culture of the mycoplasma (Okada et al., 1999).

An interesting point to remark was the association between the nasal detection of Mh at weaning with presence of EP-compatible gross lung lesions in the animals slaughtered. These results are in agreement with Fano et al. (Fano et al., 2006) who suggested that prevalence of Mh at nasal cavities of weaning pigs may be an indicator of EP-compatible lung lesions presence at slaughter-age.

In this study, serological results at slaughter indicated that infections with other pathogens (bacterial and/or viral) were also present in this herd. Concomitant presence of PRRSV, ADV, SIV and/or *A. pleuropneumoniae* in Mh infected herds has also been reported (Maes et al., 1999; Mateusen et al., 2001). In fact, Maes et al. (Maes et al., 1999) reported a significantly lower PRRSV seroprevalence in Mh vaccinated pigs. In the present study, differences in PRRSV seroprevalence were not observed between Mh vaccinated and non-vaccinated pigs; however, a reduction of serologic response to PRRSV in both vaccinated groups was observed. Although a certain protective effect of Mh vaccination on PRRSV infection has been suggested from an experimental point of view (Thacker et al., 2000), our study could not demonstrate such interaction.

The experimental design adopted in this trial probably underestimated the effect of the vaccine, since it is known that vaccination is more effective when the whole population is vaccinated than when only a part of the population is vaccinated (Guadagnini et al., 1998). The main reason to use an experimental design where all the treatment groups remained mixed was to rear all the animals with the same environmental, feeding and management conditions. However, the presence of unvaccinated pigs in the same pen/unit should increase Mh infectious pressure and, therefore, the pig's potential exposure to the agent (Andreasen et al., 2001; Dawson et al., 2002). In fact, the high prevalence of Mh in bronchus at fattening units in pigs from all groups (from 79 to 86%) suggests that the infectious pressure in the batches studied was considerably high. Therefore, under high Mh infectious pressure conditions, the used vaccine resulted in earlier seroconversion and higher percentage of seropositive pigs compared to control pigs. Moreover, reduction of EP-compatible gross lung lesions and reduction of *Mh* prevalence at upper respiratory tract sites (nasal cavity and tonsil) in slaughter pigs were observed when two doses of vaccine were administered.

Nevertheless, the present study was performed in one farrow-to-finish herd, and therefore, results obtained may not be applicable to farms with different production systems.

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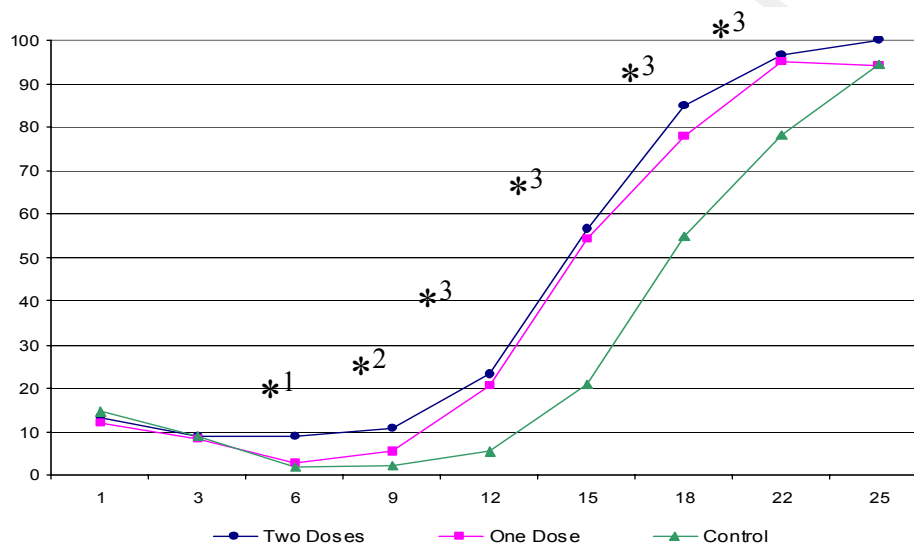
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Fig. 1: Percentage of Mh seropositive live pigs vaccinated with two doses (●) and one dose (■) of bacterin and control pigs (▲). Presence of antibodies against Mh were detected using a monoclonal blocking (ELISA CIVTEST SUIS® MYCOPLASMA HYOPNEUMONIAE, HIPRA [Girona], Spain) *¹ Statistical significant difference ($p < 0.05$) between one dose and control groups versus two dose group. *² Statistical significant difference ($p < 0.05$) between two dose group versus control group. *³ Statistical significant difference ($p < 0.05$) between one dose and two dose groups versus control group.



- 1 **Table 1:** Number (percentages) of Mh nPCR detection at nasal cavities in live animals
2 out of the total pigs tested in each sampling day.

	Weeks of age								
Treatments	1	3	6	9	12	15	18	22	25
Two doses	4/182 (2.1)	5/176 (2.8)	5/148 (3.3) ^{a,b}	12/133 (9)	33/110 (30)	28/97 (28.8)	17/78 (21.8)	26/56 (46.4)	8/31 (25.8) ^a
One Dose	3/176 (1.7)	5/162 (3)	11/140 (7.8) ^a	8/127 (6.3)	32/109 (29.3)	26/93 (27.9)	18/77 (23.3)	20/60 (33.3)	16/34 (47) ^{a,b}
Control	1/184 (0.5)	9/163 (5.5)	3/147 (2.0) ^b	8/130 (6.1)	34/113 (30)	32/98 (32.6)	24/80 (30)	19/59 (32.2)	22/36 (61.1) ^b
Total	8/542 (1.5)	19/501 (3.8)	19/435 (4.4)	28/390 (7.2)	99/332 (29.8)	86/288 (29.9)	59/235 (29.9)	65/175 (25.1)	46/101 (45.5)

- 3
4 ^{a,b} Within a column, values with different bold superscript were significantly different (*p*
5 < 0.05)

6 **Table 2:** Number (percentages) of Mh nPCR detection at nasal cavities in
 7 necropsied/slaughtered pigs out of the total pigs tested in each sampling day.
 8

	Weeks of age								
Treatments	3	6	9	12	15	18	22	25	Total
Two doses	1/13 (7.7)	1/13 (7.7)	1/13 (7.7)	2/12 (16.6)	7/16 (43.7)	7/17 (41.2)	10/19 (52.6)	8/31 (25.8) ^a	37/134 (27.6)
One Dose	0/13 (0)	0/11 (0)	1/12 (8.3)	3/13 (23)	9/15 (60)	5/17 (29.4)	5/18 (27.7)	17/34 (50) ^{a,b}	40/133 (30.1)
Control	0/11 (0)	0/12 (0)	1/12 (8.3)	3/12 (25)	8/17 (47)	5/17 (29.4)	7/17 (41.2)	21/36 (56.3) ^b	45/134 (33.5)
Total	1/37 (2.7)	1/36 (2.7)	3/37 (8.1)	8/37 (21.6)	24/48 (50)	17/51 (33.3)	22/54 (40.7)	46/101 (44.6)	122/401 (30.4)

9

10 ^{a,b} Within a column, values with different bold superscript were different ($p < 0.05$)

Table 3: Number (percentages) of Mh nPCR detection at bronchi in necropsied/slaughtered pigs out of the total pigs tested in each sampling day..

	Weeks of age								
Treatments	3	6	9	12	15	18	22	25	Total
Two doses	1/13	1/13	1/13	6/12	12/16	14/17	14/19	27/32	76/135
	(7.7)	(7.7)	(7.7)	(50)	(75)	(82)	(74)	(84)	(56)
One Dose	0/13	0/11	0/12	6/13	12/15	12/17	15/18	27/34	72/133
	(0)	(0)	(0)	(46)	(80)	(71)	(83)	(79)	(54)
Control	1/13	0/12	1/12	5/12	14/17	13/17	13/17	32/37	79/137
	(8)	(0)	(8)	(42)	(82)	(76)	(76)	(86)	(58)
Total	2/38	1/36	2/37	17/37	38/48	39/51	42/54	86/103	227/404
	(5.1)	(2.7)	(5.4)	(45.9)	(79.1)	(76.4)	(77.7)	(83.5)	(56.0)

15

16 **Table 4:** Number (percentage) of Mh nPCR detection at tonsil in
 17 necropsied/slaughtered pigs out of the total pigs tested in each sampling day.

Treatments	Weeks of age								Total
	3	6	9	12	15	18	22	25	
Two doses	1/13 (7.7)	0/13 (0)	0/13 (0)	4/12 (33.3)	10/16 (62.5)	8/17 (47.0)	12/19 (63.1)	12/32 (37.5)^a	47/135 (34.8)
One Dose	0/13 (0)	0/11 (0)	0/12 (0)	1/12 (7.7)	6/15 (40)	5/17 (29.4)	8/18 (44.4)	19/34 (55.8)^{a,b}	39/132 (29.5)
Control	0/12 (0)	1/12 (8.3)	0/11 (0)	1/12 (8.3)	8/17 (47)	5/16 (29.4)	9/17 (52.9)	26/37 (70.2)^b	50/134 (37.3)
Total	1/38 (2.6)	1/36 (2.7)	0/36 (0)	6/36 (16.6)	24/48 (50)	18/50 (36)	29/54 (55.7)	57/103 (55.3)	136/401 (33.9)

18

19 ^{a,b} Within a column, values with different bold superscript were different ($p < 0.05$)

Table 5: Median (min-max) of EP-compatible gross lung lesions (craneo-ventral pulmonary consolidation) in necropsied/slaughtered pigs. EP-compatible macroscopic lung lesion scoring was given from 1 to 5 points per lobe depending on the extension of these lesions. The maximum total score possible per lung was 35.

	Weeks of age							
Treatment	3	6	9	12	15	18	22	25
Two doses	0.00 (0-4)	0.00 (0-6)	0.00 (0-18)	0.00 (0-6)	2.32 (0-29)	4.95 (0-22)	4.50 (0-19)	2.18 (0-18) ^a
One Dose	0.00 (0-5)	0.00 (0-15)	0.00 (0-11)	0.00 (0-25)	4.85 (0-27)	5.86 (1-14)	4.41 (0-29)	2.42 (0-21) ^{a,b}
Control	0.00 (0-7)	0.00 (0-3)	0.00 (0-3)	1 (0-15)	3.57 (0-12)	3.21 (0-27)	3.17 (0-19)	5.70 (0-28) ^b

^{a,b} Within a column, values with different bold superscript were significantly different ($p < 0.05$)

Table 6: Median (min-max) EP-compatible gross lung lesions (craneo-ventral pulmonary consolidation) of necropsied and slaughtered pigs depending on the nPCR results of nasal, bronchial and tonsillar swabs. EP-compatible macroscopic lung lesion scoring was given from 1 to 5 points per lobe depending on the extension of these lesions. The maximum total score possible per lung was 35.

Localization	nPCR	n	Median Gross lung lesions (min-max)	Wilcoxon test P value
Nasal cavity	Negative	279	1.07 (0-29)	0.0003
	Positive	122	3.36 (0-28)	
Bronchus	Negative	176	0.00 (0-26)	0.0003
	Positive	225	3.99 (0-29)	
Tonsil	Negative	265	1.00 (0-29)	0.0001
	Positive	136	4.64 (0-27)	

Table 7: Number (percentages) of necropsied/slaughtered pigs showing EP-suggestive (3 or 4 histopathologic scores) microscopic lung lesions. Score 3 lesions included perivascular and peribronchiolar lymphoplasmacytic hyperplasia, pneumocyte type II hyperplasia and edema fluid in the alveolar spaces with neutrophils, macrophages and plasma cells. Score 4 was considered when score 3 lesions had evident peribronchial and perivascular lymphoid follicles.

Treatment	Weeks of age								Total
	3	6	9	12	15	18	22	25	
Two doses	0/13	3/13	6/13	6/12	9/16	13/17	18/19	28/32	80/135
	(0)	(23)	(46)	(50)	(56)	(76)	(95)	(87)	(52.2)
One Dose	0/13	2/11	5/12	4/13	8/15	16/17	17/18	31/34	83/133
	(0)	(18)	(42)	(31)	(53)	(94)	(94)	(91)	(62.4)
Control	0/13	1/12	5/12	4/12	12/17	12/17	16/17	32/37	82/137
	(0)	(8)	(41)	(33)	(70)	(71)	(94)	(86)	(59.8)
Total	0/39	6/36	16/37	14/37	29/48	41/51	51/54	91/103	245/405
	(0)	(16.6)	(43.2)	(37.8)	(60.4)	(80.3)	(94.4)	(88.3)	(60.4)