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

4
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22 **Abstract:**

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

38

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

40

41 1. Introduction

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

48 Vaccination with commercial bacterins is one of the tools used to ameliorate the
49 effects of Mh infection. Major advantages of Mh vaccination include improvement of
50 production parameters, shorter time to reach slaughter weight, reduction of clinical
51 signs and lung lesions and, occasionally, lower mortality and improvement of carcass
52 quality (Haesebrouck et al., 2004). Traditional Mh vaccination protocols include two
53 doses of bacterin, although the use of one shot vaccines is nowadays commercialized as
54 well (Baccaro et al., 2005). Vaccination with a single-dose bacterin dose has the main
55 advantage of reducing labour and causing less stress to the animals (Baccaro et al.,
56 2005; Dawson et al., 2002).

57 The efficacy of one or two doses of Mh vaccination varies depending on the
58 commercial vaccine product, schedule, farm type and the study design used (Andreasen
59 et al., 2001; Dawson et al., 2002; Kyriakis et al., 2001; Pallares et al., 2000; Ruiz et al.,
60 2002). Most of published field studies have measured the effect of vaccination against
61 Mh on lung lesion reduction and improvement of production parameters, but few of
62 them have addressed Mh infection dynamics (Baccaro et al., 2005; Martelli et al., 2006;
63 Ruiz et al., 2002).

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

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

70

71 **2. Materials and methods**

72 *2.1 Farm*

73 A one site farrow-to-finish herd with 240 sows and continuous flow production
74 system (in nursery and finishing units) was selected. The farm had respiratory clinical
75 signs in fattening pigs (coughing) and EP-compatible lung lesions at slaughter. To
76 confirm Mh-related disease, a nPCR from nasal swabs and evaluation of gross lung
77 lesions from 34 slaughtered pigs were performed. Moreover, blood samples from other
78 21 slaughtered pigs were also taken. All 34 (100%) tested pigs were positive to Mh by
79 nPCR and had a mean macroscopic lung score of 9.16 based on the classification by
80 Hannan et al. (Hannan et al., 1982). On the other hand, 13 out of 21 (62%) were
81 seropositive to Mh.

82

83 *2.2 Study design*

84 This was a blinded, randomized and controlled field trial. A total of 542 pigs
85 (268 female and 274 male) from 69 different sows of 6 different farrowing batches were
86 included in this study. The parity range of selected sows was from 1 (n=5) to 8 (n=2),
87 with a mean of 4.4 parity number. No Mh vaccination or medication protocols were
88 being performed before the start of the study. Piglets were ear-tagged at 1 week of age
89 and randomly distributed in the following treatment groups: 1) Two dose group
90 (n=182): pigs that received 2 ml of vaccine at 1 and 3 weeks of age and 2 ml of

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

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

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

117

118 2.3 Sample collection

119 Nasal swabs (Culturette TM, Becton Dickinson, Sparks, USA) and blood samples (5 ml
120 Venoject, Terumo Europe, Madrid, Spain) from all animals were taken at 1, 3, 6, 9, 12,
121 15, 18, 22 and 25 weeks of age. Nasal swabs were obtained by swabbing the nasal
122 mucosa of both nostrils reaching deeply into turbinates. Moreover, blood sample from
123 sows was collected at 1 week post-farrowing (the same day as piglet sampling at 1
124 weeks of age).

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

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

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

150

151 *2.4 DNA extraction and nPCR to detect Mh nucleic acid.*

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

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

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

167

168 2.5 ELISA technique to detect Mh antibodies

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

$$178 \quad \text{IP (\%)} = \frac{\text{Mean negative value OD} - \text{sample OD}}{\text{Mean negative value OD}} \times 100$$

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

189

190 *2.6 Pathological studies*

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

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

212

213 *2.7 Statistical analyses*

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

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

228 Statistical analyses were performed with the SAS system for windows version
229 8.0 (SAS Institute Inc, Cary, North Carolina, USA). Statistical significance level was set
230 at $\alpha=0.05$.

231 The vaccine efficacy in reducing lung score was calculated, using group median
232 lung scores (Jones et al., 2005), as follows: Vaccine efficacy = [(median non-vaccinated
233 group score – median vaccinated group score)/ median non-vaccinated group score] x
234 100.

235

236 **3. Results**

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

239 sows neither per treatment nor per parity number were found. On the contrary, sow
240 parity number was significantly associated with serologic status of piglets at 1 one week
241 of age ($p=0.006$). At that age, all piglets ($n=35$) coming from gilts ($n=5$) were *Mh*
242 seronegative. Nevertheless, it should be taken into account that the five gilts included
243 in this study were seronegative. On the other hand, sow parity number was also
244 significantly related with detection of *Mh* in their piglets by PCR at 9 ($p=0.003$) and 12
245 ($p=0.0004$) weeks of age. Specifically, was detected in higher percentages in piglets
246 coming from gilts (5 out of 24 [20.8%] and 12 out 19 [63.1%] at 9 and 12 weeks of age
247 respectively) than from sows of intermediate (12 out of 263 [4.5%] and 69 out 220
248 [31.3%], at 9 and 12 weeks of age respectively) or high (11 out of 105 [10.4%] and 18
249 out 94 [19.1%], at 9 and 12 weeks of age respectively) parity number.

250 Globally, *Mh* nPCR detection percentages at nasal cavities were very similar in
251 all three treatment groups (Table 1). From 1 to 9 weeks of age, *Mh* was detected in
252 nasal cavities in relatively low proportion ($<10\%$) in all groups. On the contrary, *Mh*
253 detection percentages tended to increase in all three treatments groups from 12 weeks of
254 age until the slaughter age. At 6 weeks of age, there were higher percentages of pigs
255 positive by nPCR in one dose group than the control group of pigs ($p < 0.05$).
256 Moreover, at slaughter age, percentage of nPCR positive pigs at nasal cavities was
257 higher in the group of animals that received two doses of vaccine than in control pigs (p
258 < 0.05).

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

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

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

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

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

289 affected, 36 (8.9%) showed between 25 to 50% (17.5 out of 35 scoring points) damaged
290 lung, 18 (4.5%) with 50 to 75% (26.25 out of 35 scoring points) lung affected and,
291 finally, 5 (1.2%) had more than 75% of the lung affected. No gross lung lesions
292 compatible with EP were assessed in one pig due to a severe, diffuse fibrous pleuritis
293 observed at the moment of necropsy. Although EP-compatible gross lung lesions
294 (pulmonary craneo-ventral consolidation) were observed in animals of different ages
295 and treatments, the extension of these lesions increased at 12-15 weeks of age onwards
296 (Table 5). At 25 weeks of age, median lesional scores were lower in two-dose
297 vaccinated pigs (score value of 2.18) and one-dose vaccinated animals (score value of
298 2.42) compared to control pigs (score value of 5.70); however, only significant
299 differences ($p=0.05$) were confirmed between the two dose and the control groups.
300 Vaccine efficacy in reducing lung lesions score was 61.8% and 57.8% when comparing
301 two and one dose vaccination groups, respectively, with controls animals.

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

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

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

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

324

325 **4. Discussion**

326 The present study describes the pattern of Mh infection and seroconversion in
327 vaccinated and non-vaccinated pigs in a farrow-to-finish operation with EP associated
328 clinical signs. Monitoring pigs from birth to slaughter with sequential necropsies every
329 3 weeks allowed understanding the effect of different vaccination schedules on
330 localization of Mh in different respiratory tract sites and EP lung lesion evolution.

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

339 controls pigs. Similar or lower lung lesion reduction has been also found in other field
340 studies using two doses of Mh vaccine (Kyriakis et al., 2001; Maes et al., 1999; Scheidt
341 et al., 1994). On the other hand, single vaccination at 6 weeks of age resulted in 57.8%
342 lung lesion reduction; however, the extent of these lesions were not statistical
343 significantly different from the control group, probably due to relatively low mean lung
344 lesional score observed (4.17 versus 7.71, respectively). Obtained results for single Mh
345 vaccination are in the same line than those achieved in other studies (Baccaro et al.,
346 2005; Dawson et al., 2002) in which vaccination was performed at weaning age. On the
347 contrary, two field studies in which one dose vaccine was administered at 10 weeks of
348 age (Kyriakis et al., 2001; Pommier et al., 2000) found relatively low (<20%) lung
349 lesion reduction, probably due to the short time between vaccine application and timing
350 of infection. In the present study, single vaccination was administered approximately 6
351 to 9 weeks before most of the pigs had Mh at bronchus, suggesting that pigs had enough
352 time to develop a protective immune response able to reduce the extent of gross lung
353 lesions.

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

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

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

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

377 Under field conditions, seroconversion to Mh is usually quite delayed and occurs
378 mainly in growing and finishing units (Leon et al., 2001). In the present study,
379 seroconversion was observed 3 weeks before in both vaccinated groups (between 9 to
380 12 weeks of age) than in control animals (between 12 to 15 weeks of age). Both
381 vaccination groups had a very similar serumprofile, only differing statistically at 6
382 weeks of age. At that week, percentage of seropositive pigs in the two dose group did
383 not decline to the low percentage as the one dose group did, indicating a humoral
384 immune response due to vaccination. On the other hand, at 12 weeks of age, percentage
385 of seropositive pigs from one dose group reached nearly the same percentage as the two
386 dose group (21 and 23%, respectively). Therefore, obtained results indicated the
387 capability of both vaccine schedules to elicit a detectable humoral immune response to
388 Mh after vaccination.

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

398 Detection of the pathogen in all treatment groups and ages confirmed that Mh
399 vaccination did not prevent from infection (Maes et al., 1999); however, vaccination
400 was related with a lower Mh prevalence at slaughter age in upper respiratory airways
401 (nasal cavities and tonsils), as previously described (Baccaro et al., 2005; Ruiz et al.,
402 2002). This result is probably due to a reduction of Mh excretion (Baccaro et al., 2005).
403 The fact that no differences in Mh prevalence at bronchus were observed may be related
404 to the qualitative technique used to detect this pathogen. It is possible that vaccinated
405 pigs may have lower Mh load at bronchus and, consequently, may shed less pathogen
406 particles (detectable at the upper respiratory tract) than control pigs. A quantitative
407 method for Mh detection would help to confirm if vaccination reduces bacterial load at
408 bronchus, as previously found by culture of the mycoplasma (Okada et al., 1999).

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

414 In this study, serological results at slaughter indicated that infections with other
415 pathogens (bacterial and/or viral) were also present in this herd. Concomitant presence
416 of PRRSV, ADV, SIV and/or *A. pleuropneumoniae* in Mh infected herds has also been
417 reported (Maes et al., 1999; Mateusen et al., 2001). In fact, Maes et al. (Maes et al.,
418 1999) reported a significantly lower PRRSV seroprevalence in Mh vaccinated pigs. In
419 the present study, differences in PRRSV seroprevalence were not observed between Mh
420 vaccinated and non-vaccinated pigs; however, a reduction of serologic response to
421 PRRSV in both vaccinated groups was observed. Although a certain protective effect of
422 Mh vaccination on PRRSV infection has been suggested from an experimental point of
423 view (Thacker et al., 2000), our study could not demonstrate such interaction.

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

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

442

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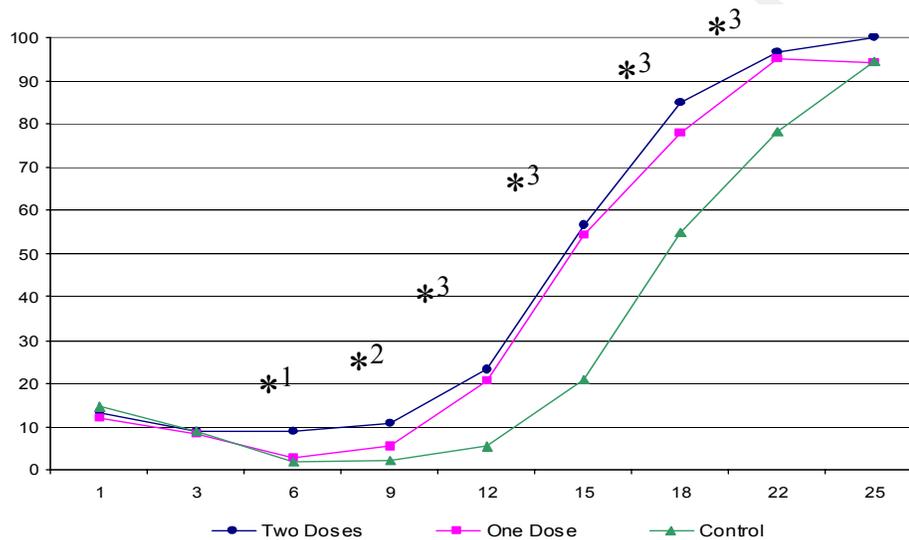
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Fig. 1: Percentage of Mh seropositive live pigs vaccinated with two doses (●) and one dose (■) of bacterin and control pigs (▲). Presence of antibodies against Mh were detected using a monoclonal blocking (ELISA CIVTEST SUIS® MYCOPLASMA HYOPNEUMONIAE, 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.

Treatments	Weeks of age								
	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 ($p < 0.05$)

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

13

	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)

14

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 ($p < 0.05$)

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

24

	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

25 ^{a,b} Within a column, values with different bold superscript were significantly different (p

26 < 0.05)

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

32

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)	

33

34 **Table 7:** Number (percentages) of necropsied/slaughtered pigs showing EP-suggestive
 35 (3 or 4 histopathologic scores) microscopic lung lesions. Score 3 lesions included
 36 perivascular and peribronchiolar lymphoplasmacytic hyperplasia, pneumocyte type II
 37 hyperplasia and edema fluid in the alveolar spaces with neutrophils, macrophages and
 38 plasma cells. Score 4 was considered when score 3 lesions had evident peribronchial
 39 and perivascular lymphoid follicles.

40

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

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42

43