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SHORT COMMUNICATION

**SEROLOGICAL, PATHOLOGICAL AND POLYMERASE CHAIN REACTION STUDIES ON
MYCOPLASMA HYOPNEUMONIAE INFECTION IN THE WILD BOAR**

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26 **Abstract**

27 There are evidences that wild boar and domestic pig populations share the vulnerability to certain
28 pathogens and, in consequence, the risk of pathogen transmission. Based on serological evidences, it is
29 known that *Mycoplasma hyopneumoniae* (Mhyo) is able to infect the wild boar. However, if this
30 infection causes lesions and disease in this species is still unknown. The objectives of the present study
31 were to assess the seroprevalence to Mhyo, as well as the rates of detection of Mhyo (by nested
32 polymerase chain reaction, nPCR) in upper and lower respiratory tract of the wild boar. Moreover,
33 presence of enzootic pneumonia (EP)-like gross and microscopic lesions in these animals was also
34 assessed. Antibodies against Mhyo were detected in 92 out of 428 (21%) serum samples tested.
35 Moreover, Mhyo DNA was detected by nPCR in 17 out of 85 (20%) nasal swabs and in 12 out of 156
36 (8%) lung samples. No gross EP-like lesions were observed in any of the studied pigs. Besides, presence
37 of EP-like microscopic lung lesions was observed in 18 out of 63 (29%) animals. Mhyo DNA was
38 detected at lung level in 2 out of these 18 (11%) animals. Presence of EP-like microscopic lesions was
39 significantly related ($p<0.05$) with Mhyo detection at nasal swab swab but not ($p>0.05$) with its
40 detection in lung samples; such lesions were also linked ($p<0.05$) with presence of *Metastrongylus* spp-
41 like parasite structures. Results of the present study confirm that Mhyo is able to infect and might be
42 able to cause EP-like microscopic lesions in wild boar but, apparently, with a subclinical impact.

43

44 Key words:

45 *Mycoplasma hyopneumoniae*, wild boar, seroprevalence, infection, enzootic pneumonia

46

47

48

49 **1. Introduction**

50 Populations of the Eurasian wild boar (*Sus scrofa*) have been expanding throughout Europe
51 during last decades (Acevedo et al., 2006). Consequently, probabilities of interaction between wild boar
52 and domestic pigs as well as pathogen transmission are also increased. Therefore, the study of the
53 prevalence of pathogens in wild boar populations and the role of these animals as pathogen reservoirs is
54 of importance for public health and pig producers (Ruiz-Fons et al., 2008; Wyckoff et al., 2009). There
55 are evidences that domestic and wild boar share the vulnerability to certain viral and bacterial pathogens
56 (Ruiz-Fons et al., 2008; Wyckoff et al., 2009). However, little information on the prevalence and impact
57 of *Mycoplasma hyopneumoniae* (Mhyo), one of the most economically important pathogens for the pig
58 industry, is available for the wild boar.

59 Mhyo is the etiologic agent of enzootic pneumonia (EP), a chronic respiratory disease with high
60 morbidity and low mortality. The most significant clinical sign of EP is a non-productive cough
61 (particularly in finishing pigs) and the main gross associated lesion is a craneo-ventral pulmonary
62 consolidation (Caswell and Williams, 2007). Histologically, EP-like lesions are characterized by a
63 broncho-interstitial pneumonia with broncho-alveolar lymphoid tissue (BALT) hyperplasia (Caswell and
64 Williams, 2007).

65 Up to now, evidences of infection (Marois et al., 2006) and detection of antibodies against Mhyo
66 (Marois et al., 2006, Vengust et al., 2006, Shcherbakov et al., 2007) have been reported in different
67 countries. However, it is unknown if this pathogen is able to cause EP-like lesions to the wild boar.
68 Therefore, the aims of this study were firstly to gain insight into Mhyo seroprevalence, infection and
69 excretion in the Spanish wild boar population and, secondly, to assess the potential presence of EP-like
70 lesions in these animals.

71

72 2. Materials and methods

73 2.1. Animals and samples

74 Number of samples tested and techniques performed are summarised in table 1. A total of 428
75 wild boar serum samples (males, n=185; females n=231; non-recorded gender, n=12) were included in
76 this study. Tested sera were obtained from piglets (<7 months of age, n=3), weaners (7-12 months,
77 n=95), juveniles (12-24 months, n=87) and adult wild boars (>24 month, n=234). Age was unknown for
78 9 animals. Age classification was based on tooth eruption patterns and weight (Sáenz De Buruaga et al.,
79 1991). From these 428 samples, 225 sera were collected during the hunting season between years 2000
80 and 2008 in the South-Central part of Spain. Samples from this region were collected from hunter-
81 harvested wild boar reared in high-wire fenced hunting estates. Rest of the samples (n=203) were
82 collected from wild boars in North-Eastern Spain between 2004 and 2005. Samples from this region
83 were collected from hunted free-living wild boars in national parks. Fresh lung samples were also taken
84 from 156 (102 from South-Central and 54 from North-Eastern Spain) out of these 428 wild boars, and
85 used to assess Mhyo infection at the lower respiratory tract. Moreover, different portions of lung were
86 taken from 63 (all of them from South-Central Spain) out of these 156 animals, and fixed by immersion
87 in formalin to perform histopathological studies. None of these 63 animals showed gross lesions
88 attributable to Mhyo (pulmonary cranio-ventral consolidation). Finally, presence of this pathogen in the
89 upper respiratory tract (nasal swabs) was also assessed in 85 (all of them from South-Central Spain) out
90 of these 156 animals.

91

92 2.2. Serology

93 Sera were tested by a monoclonal blocking ELISA (Laboratorios HIPRA[®], Girona, Spain) to
94 detect Mhyo antibodies. The inhibition percentage (IP) was calculated taking into account the optical
95 densities (OD) of each sample as well as the one of the negative control (negative value). ELISA result

96 interpretation, based on the percentage of inhibition (IP) values, was as follows: IP < 50%, negative and
97 IP > 50%, positive.

98

99 **2.3. DNA extraction and PCR methods**

100 Nasal swabs were suspended in 1 ml of sterile PBS and vigorously vortexed. Nasal swab
101 suspensions and fresh lung tissue were stored at -80°C until further processing. DNA was extracted
102 using 300 µl of nasal swab suspensions (Nucleospin® Blood, Macherey-Nagel GmbH & Co KG Düren,
103 Germany) and from 25 mg (approximately) of fresh lung sample (Nucleospin® Tissue, Tissue Macherey-
104 Nagel GmbH & Co KG Düren, Germany), according to manufacturer's instructions. To assess for
105 potential contaminations during the extraction procedure, a negative control was included using PBS as
106 extraction substrate in each group of extracted samples. Presence of Mhyo DNA was studied using a
107 previously described nested PCR (nPCR) (Calsamiglia et al., 1999). In each nPCR reaction, a negative
108 control (consisting of millipore water instead of extracted DNA) was added every four test samples.
109 DNA extracted from a pure culture of Mhyo was used as positive control and included in the last
110 position of the gel.

111

112 **2.4. Histopathology**

113 Since no gross EP-like lesions were evident, portions of the cranial part of the apical, medium
114 and diaphragmatic lobes were collected and fixed by immersion in 10% buffered formalin. Lung
115 samples were processed routinely for histopathology (haematoxylin&eosin stain). Presence of
116 microscopic lung lesions were graded from 0 to 4, following previously described criteria (Calsamiglia
117 et al., 2000). Lesions scoring 3 and 4 were considered compatible with EP. Presence (or absence) of
118 *Metastrongylus spp*-like parasites (eggs, larvae and/or adult individuals) (Caswell and Williams, 2007)
119 in lungs sections was recorded.

120

121 2.5. Statistical analyses

122 Bivariate analyses using contingency tables (Chi-square statistics) were used to compare: 1)
123 serology, Mhyo DNA detection by nPCR (nasal swab and lung tissue) and presence of EP-like
124 microscopic lesions (histopathological scores 3 or 4) taking into account origin, sex and age of the
125 animals, 2) serology versus nPCR detection (nasal swab and lung tissue) and EP-like microscopic
126 lesions, and 3) EP-like microscopic lesions versus nPCR detection (nasal swab and lung tissue), and 4)
127 presence of *Metastrongylus spp-like parasites* against the rest of studied parameters. To analyze the
128 relationship between serology and nPCR results versus lung lesions, animals were divided in non-EP-
129 like (histopathological score 0 to 2) or EP-like (scoring 3 or 4) microscopic lung lesions. Relationship
130 between nPCR detection at nasal swabs and lung samples was assessed by means of the McNemar test
131 and the Kappa value. Statistical analyses were performed with the SAS system for Windows version 9.0
132 (SAS Institute Inc, Cary, North Carolina, USA). Statistical significance level was set at $\alpha=0.05$.

134 3. Results

135 Number of tested samples per technique and number of samples that yielded positive results are
136 detailed in table 1.

137 A total of 428 serum samples were analyzed by ELISA. Ninety two out of the 428 (21.5%) sera
138 were Mhyo seropositive. No statistically significant differences ($p>0.05$) between number of Mhyo
139 seropositive from North-Eastern (36 out of 203, 18%) and South-Central (56 out of 225, 25%) parts of
140 Spain were found. The highest number of Mhyo seropositive animals were observed in the adult
141 population ($n=60$ [25%], $p<0.05$) (table 2). No statistically significant differences ($p>0.05$) between
142 numbers of Mhyo seropositive males (41%) and females (53%) were observed (table 3).

143 Mhyo DNA was detected by nPCR in 12 (8%) out of the 156 analyzed lung samples. No
144 statistically significant differences ($p>0.05$) between number of nPCR positive lung samples from
145 animals hunted in North-Eastern (4 out of 54, 7%) and South-Central (8 out of 102, 8%) parts of Spain

146 were found. Number of Mhyo seropositive animals with an nPCR positive lung tissue (n=10 [83%]) was
147 significantly higher ($p<0.05$) than the number of seronegative ones (n=2 [17%]). No statistically
148 significant differences in the number of nPCR positive lung samples regarding age and gender of tested
149 animals were found.

150 From the 85 animals from which nasal swab and lung sample were available, Mhyo was detected
151 by nPCR in lung only in 2 (2%) animals, in nasal swab only in 13 (15%) animals, and in both samples in
152 4 (5%) animals (McNemar test $p<0.004$, Kappa= 0.27).

153 Number of Mhyo seropositive animals with an nPCR positive nasal swab (n=11 [65%]) was
154 significantly higher ($p<0.05$) than the number of seronegative ones (n=6 [35%]). No statistically
155 significant differences in the number of nPCR positive nasal swabs regarding age (table 2) and gender
156 (table 3) were found among tested animals.

157 Eighteen out of the 63 (29%) lung samples evaluated by histopathology showed EP-like
158 microscopic lesions (10 lungs scored 3 and 8 scored 4). Nasal swab was available in 15 out of these 18
159 animals showing EP-compatible lung lesions; Mhyo DNA was detected in 9 out of these 15 (60%) nasal
160 swabs ($p<0.05$). On the other hand, Mhyo was detected at lung level only in 2 out of the 18 (11%)
161 animals showing EP-like microscopic lesions. Mhyo was detected in nasal swab and lung at the same
162 time in these 2 animals. No statistically significant differences between seropositive (6 out of 18) and
163 seronegative (12 out of 18) animals showing EP-like microscopic lesions ($p>0.05$) were found. Also, no
164 statistically significant differences in the number of animals showing EP-like microscopic lesions
165 regarding age and gender of the tested animals were found.

166 Presence of *Metastrongylus* spp.-like parasite structures were observed in 6 (33.3%) out of these
167 18 animals showing EP-compatible lung lesions ($p<0.05$). On the contrary, parasites were observed in
168 only 2 (4%) out of the 45 animals that did not show these lesions. All these 8 wild boar harbouring
169 parasites were negative by Mhyo nPCR at lung level and only 2 (28.5%, $p>0.05$) were positive by nPCR
170 in nasal swabs (available in 7 out of these 8 animals). No statistically significant differences in the

171 number of animals showing presence of *Metastrongylus* spp.-like parasite structures regarding age and
172 gender of the tested animals were found.

173 From the 54 animals from which all the samples were available, one (2%) wild boar was Mhyo
174 seropositive, nPCR positive (at nasal as well as at lung levels) and had EP-like microscopic lesions with
175 no evidence of parasitosis.

176

177 **4. Discussion**

178 The present study gives further light on the role of Mhyo infection in the wild boar. Presence of
179 antibodies against this pathogen, together with the detection of its DNA and presence of
180 histopathological lesions compatible with EP suggest that, besides the infection, Mhyo is capable of
181 causing broncho-interstitial pneumonia in wild boar. However, lack of consistent gross lesions
182 compatible with EP in studied animals points out that the effect of Mhyo probably remains subclinical in
183 this species. Taking into account that a number of wild boar had the pathogen in the nose, its potential
184 role as a reservoir for the domestic pig can not be ruled out. Although wild boars specifically included in
185 this study came from areas in which the contact with domestic pigs was unlikely, the putative
186 transmission between wild boars and domestic pigs should not be considered negligible and an especial
187 risk for Mhyo seronegative farms. These type of farms tend to be high health herds, usually isolated
188 from high density pig rearing areas (Muirhead and Alexander, 1997) and, therefore, close to areas where
189 wild boar live.

190 Results of this study demonstrated the presence of Mhyo seropositive animals in more than 400
191 wild boars from different Spanish regions. Prevalence of Mhyo seropositive pigs obtained in this study
192 (21%) was similar to the one obtained in another serologic survey conducted in Slovenia (21%)
193 (Vengust et al., 2006), but lower to the one observed in France (58%) (Marois et al., 2006). On the
194 contrary, these results are in disagreement with a previous epidemiologic study conducted in South-
195 Central Spain, in which 78 wild boar (hunted between 1998 and 2000) were seronegative against this

196 respiratory pathogen (Vicente et al., 2002). Differences in geographical origin, year of collection as well
197 as number of samples could explain these discrepancies. However, the fact that the tested samples were
198 collected in two different regions in different years makes difficult to assess potential differences in
199 seroprevalence and rate of infection between the two areas studied.

200 Detection of Mhyo infection in wild boar was already described by Marois et al. (Marois et al.,
201 2006), who detected 15 out of 171 lung exudates positive by PCR. However, detection of Mhyo DNA in
202 that study was not successfully confirmed by culture and presence of lung lesions was not assessed. In
203 the present study, infectiousness of Mhyo PCR positive material was not tested; therefore, like in Marois
204 et al. (2006), the achievement of a Mhyo isolate from wild boar is still lacking. However, the present
205 study represents the first description of Mhyo infection in wild boar at different levels of the respiratory
206 tract (upper and lower levels) in animals showing microscopic lung lesions compatible with EP. It
207 should be taken into account, however, that other agents such as viruses (SIV) or parasites
208 (*Metastrongylus* spp.) might also cause broncho-interstitial pneumonia with BALT hyperplasia (Caswell
209 and Williams, 2007). In fact, some animals had lesions probably attributed to *Metastrongylus* spp
210 infestation, since parasite structures were observed by histopathology in Mhyo seronegative and PCR
211 negative hosts. Moreover, infestation was related to the presence of broncho-interstitial pneumonia. It
212 can not be ruled out, therefore, that EP-like microscopic lesions in wild boar with no evidence of
213 parasitisation were indeed infested with *Metastrongylus* spp. On the other hand, it is worthy to remark the
214 existence of one Mhyo seropositive animal infected with this pathogen at upper and lower respiratory
215 tract and with EP-compatible lung lesions and no evidence of parasitisation.

216 In the present study, Mhyo detection in nasal swabs was linked to the presence of EP-like
217 microscopic lesions and to nPCR detection in lung samples, similarly to what has been found for the
218 domestic swine at slaughter (Sibila et al., 2004a). On the contrary, detection of Mhyo DNA by nPCR in
219 lung tissue was not linked to the presence of EP-like microscopic lesions. This result would be in

220 disagreement with previously published works in domestic pigs, in which Mhyo was detected in lung
221 tissues in a high percentage of cases with moderate to severe lung lesions (Cai et al., 2007; Moorkamp et
222 al., 2007). The low detection rate of Mhyo in lung tissues might be attributable to several lung lesions
223 observed were not caused by Mhyo and, therefore, this pathogen was not located in lungs, even it was
224 present in the wild boar environment (it was detected in the nose). Secondly, different portions of tissue
225 were tested by nPCR and histopathology. In fact, none of the 63 animals from which lung samples were
226 available showed gross lung lesions and, thus, the portion of tissue processed by nPCR was taken
227 without any reference of lesion. Finally, presence of EP-like microscopic lesions may depend, among
228 other factors, on the infectious pressure (Sibila et al., 2009). Since wild boar tend to live in open-air
229 systems; therefore, the infectious pressure must be considered lower than the one found in intensively
230 reared pig farms and may be not sufficient to cause typical EP-like gross lesions. On the other hand,
231 absence of macroscopic lesions attributable to Mhyo in domestic pigs showing EP-like microscopic
232 lesions has been already described elsewhere (Sibila et al., 2007) and may indicate a chronic stage of
233 Mhyo infection, where gross lesions had healed but microscopic lesions remained.

234 In summary, results from this study further expand the existing knowledge on Mhyo infection in
235 the wild boar population, pointing out that Mhyo might be able to cause EP-like microscopic lesions in
236 wild boar, even they seem to have a subclinical impact. Based on the relatively high prevalence of Mhyo
237 in conventional pig farms (Sibila et al., 2004b), it should not be ruled out the fact that domestic pig can
238 be the real reservoir for the wild boar. The wild boar may only represent a potential Mhyo threaten for
239 Mhyo free farms.

240

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243 samples for histopathology.

244

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Table 1: Combination of the different techniques performed in the same animal (rows) according to the number of positive samples per technique (rows). nPCR NS (nPCR nasal swabs); nPCR L (nPCR Lung); Histo (Histopathology, animals scored 3 and 4).

		Number of tested animals	Number of positive samples (percentage) per technique				
			Serology	nPCR		Histo*	Presence of <i>Metastrongylus</i> spp.-like structures
				NS	L		
Techniques performed in the same animal	Serology	272	33 (12)				
	Serology nPCR L	62	29 (47)		5 (8)		
	Serology nPCR NS nPCR L	31	8 (26)	3 (10)	2 (6)		
	Serology nPCR L Histo	9	4 (33)		1 (11)	3 (33)	1 (11)
	Serology nPCR NS nPCR L Histo	54	18 (33)	14 (26)	4 (7)	15 (28)	8 (15)
	Total	428	92 (21,5)	17 (20)	12 (8)	18 (29)	9 (14)

Table 2: Results of the different techniques in regards the age of the tested wild boar. nPCR NS (nPCR nasal swabs); nPCR L (nPCR Lung); Histo (Histopathology, animals scored 3 and 4).

Age groups	ELISA		nPCR NS		nPCR L		Histo (score 3 or 4)		Metastrongylus	
	N	Positive	N	Positive	N	Positive	N	Positive	N	Positive
Piglets	3	0	0	0	0	0	0	0	0	2
Weaners	95	17	17	5	29	5	10	3	10	2
Juveniles	87	11	20	2	28	0	12	6	12	34
Adults	234	60	47	10	90	7	34	7	34	7
Unknown	9	4	1	0	9	0	7	2	7	2
Total	428	92	85	17	156	12	63	18	63	8

Table 3: Results of the different techniques by sex of the tested wild boar. nPCR NS (nPCR nasal swabs); nPCR L (nPCR Lung); Histo (Histopathology, animals scored 3 and 4).

	ELISA		nPCR NS		nPCR L		Histo (score 3 or 4)		Metastrongylus	
	N	Positive	N	Positive	N	Positive	N	Positive	N	Positive
Male	185	38	32	6	64	8	28	8	28	2
Female	231	49	49	10	87	4	33	10	33	6
Unknown	12	5	4	1	5	0	2	0	2	0
Total	428	92	85	17	156	12	63	18	63	8