

Transmission of pathogenic respiratory bacteria to specific pathogen free pigs at slaughter.

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30 Our results suggest that the SPF pigs became contaminated mainly by the slaughterhouse
31 environment and the scalding water. Histological examinations revealed that during scalding,
32 contaminated water could reach the trachea and the lungs of pigs. Checks conducted at
33 slaughter for respiratory disorders have to be carried on, but nasal cavities and tonsils are
34 not appropriate for bacteriological investigations. Moreover, bacteriological results obtained
35 from the lungs of slaughtered pigs have to be used with carefulness.

36

37 **Keywords** : SPF pigs, slaughterhouse, scalding tank water, pathogenic respiratory bacteria

38

39 **1. Introduction**

40 *Mycoplasma hyopneumoniae*, a widespread respiratory pathogen in pigs, is the primary
41 agent of swine enzootic pneumonia. In combination with various other infectious agents,
42 such as bacteria and viruses, this chronic disease is implicated in a syndrome known as the
43 porcine respiratory disease complex (PRDC) causing substantial losses to the pig industry
44 (Thacker et al., 1999; Thacker et al., 2001; Opriessnig et al., 2004; Thanawongnuwech et al.,
45 2004). Among the pathogenic respiratory bacteria, *Actinobacillus pleuropneumoniae*,
46 *Pasteurella multocida*, *Haemophilus parasuis* and *Streptococcus suis* are commonly
47 associated with *M. hyopneumoniae* and can induce pathologic alterations in the thoracic
48 organs of the pig. Lesions such as pneumonia, pleuropneumonia, pleuritis, abscesses and
49 pericarditis can be observed in growing pigs during slaughter examinations that are an
50 important diagnostic tool for surveillance of herd health at regular intervals. Microbiological
51 investigations have been performed on slaughter. Detection of pathogenic bacteria involved
52 in the lung lesions is the first step to control and to prevent respiratory disorders in pig herds
53 (Pijoan and Fuentes, 1987; Andreasen et al., 2001). Pathogenic respiratory bacteria are
54 generally detected by culture or PCR from the most effective samples carried out for each
55 bacterial species, after macroscopic examinations and quantification of the extend of lung
56 lesions (Calsamiglia et al., 1999; Schaller et al., 2001; Oliveira et al., 2001; Marois et al.,
57 2004).

58 It is widely accepted that respiratory pathogens are transmitted by direct contact from pig to
59 pig or by coughing and sneezing of infected pigs in close proximity. Pathogen circulation can
60 also occur from infected to naïve pigs, in short distances by airborne transmission (Otake et
61 al., 2002; Thacker, 2006; Gottschalk and Taylor, 2006).

62 At the end of the fattening period, pigs are collected from different farms and transported to
63 the slaughterhouse, sometimes in the same vehicle. They wait in lairage at the
64 slaughterhouse before slaughtering. The lairage duration is carried out during at least three
65 hours, the time required before slaughtering for animal welfare and meat quality reasons.

66 To our knowledge, unlike bacterial species isolated from the digestive tract, no information is
67 available about the risk factors influencing the spread of respiratory pathogens at slaughter.

68 The main objectives of this work were to study (1) the impact of contact between pigs before
69 slaughtering on transmission of pathogenic bacteria from the respiratory tract of conventional
70 finishing pigs to Specific Pathogen Free (SPF) pigs, (2) the samples allowing the recovery of
71 the pathogenic respiratory bacteria from the SPF pigs and (3) the role of the slaughterhouse
72 environment and of the scalding tank water.

73

74 **2. Materials and methods**

75 *2.1. Animals*

76 Thirteen 5 month-old hysterectomy derived SPF pigs were obtained from the porcine
77 experimental unit of AFSSA Ploufragan (Agreement B-22-745-1). Very strict biosecurity
78 measures were implemented in order to avoid undesirable contamination of the pigs: air
79 filtration system and airlocks for each unit, unit-specific clothes and compulsory showering
80 before and after visiting the pigs (Cariolet et al., 1994).

81 The selected SPF pigs were ear tagged and carried to the slaughterhouse in a cleaned and
82 disinfected conventional lorry. Forty-eight hours before departure, bacteriological analyses of
83 the respiratory tract were performed on the animals. Drag-swab samples were also taken
84 from different places in the lorry.

85 A first group of nine SPF pigs (average weight: 114.6 kg (\pm 9.9 kg)) was brought, very early
86 in the morning, just before the onset of slaughter, to a cleaned commercial slaughterhouse,

87 where they were in contact during 4 h with finishing pigs coming from several conventional
88 commercial herds. One week later, a second group of four SPF pigs (average weight: 111 kg
89 (\pm 16.5 kg)) was slaughtered 4 h after the onset of slaughter. Pigs of group 2 were
90 slaughtered immediately at arrival, without any contact with conventional finishing pigs.

91

92 *2.2. Samples*

93 *2.2.1. SPF pigs*

94 Before departure for the slaughterhouse, the SPF pigs were swabbed. CytoBrushes
95 (VWR International, Fontenay-sous-Bois, France) were used for nasal cavities and palatine
96 tonsils. Sterile catheters (Laboratoire Euromedis, Neuilly sous Clermont, France) were used
97 for trachea swabbing (Marois et al., 2007). Drag-swabs (Sodibox, La Forêt Fouesnant,
98 France), humidified with 5 mL of Buffered Pepton Water Broth (BP), were also taken from
99 different places in the lorry.

100 At the end of the slaughterline, the thoracic organs of the SPF pigs were checked,
101 especially for respiratory disorders. Nasal cavities and tonsils were swabbed. Lungs with
102 trachea and whole palatine tonsils were recovered and carried to our laboratory. Trachea
103 was swabbed with a CytoBrush. Whole tonsils and pieces of the right middle lobe of lungs
104 were cauterised on the surface with a hot spatula and swabbed after several parallel
105 incisions. Each swab was placed in 2 mL of BP (Initial Suspension: IS). The drag-swabs
106 were placed in 10 mL of BP and the supernatant were centrifuged at 5.000 x g for 45 min.
107 The pellet was resuspended into 2 mL of BP (IS).

108

109 *2.2.2. Scalding tank water*

110 Just before slaughtering of the SPF pigs, 50 mL water samples from the scalding tank were
111 collected, with sterile bottles, thrice at a depth of 0.20 m, before the onset of slaughter (T0),
112 as soon as the SPF pigs arrived (T+4 h) and 4 h later (T+8 h), at 5 different places (every 10
113 m) in the tank (50 m long and 1 m wide). In this slaughterhouse, the scalding tank was
114 washed and cleaned every day. The temperature of the scalding water was measured during
115 each sampling.

116

117 2.3. Post-mortem examinations of finishing pigs from conventional herds

118 In order to have some information about the score of lung lesions during the day of slaughter,
119 post-mortem examinations were carried out on 320 finishing pigs slaughtered one hour
120 before the SPF pigs. The lung lesions were scored according to the method previously
121 described by Madec and Derrien (1981). According to this method, the maximum total score
122 possible for each lung was 28.

123

124 2.4. Bacteriological investigations

125 Samples were prepared for PCR assays as described by Kellog and Kwok (1990).
126 Briefly, 1 mL of each IS was centrifuged ($12.000 \times g$, 4°C, 20 min) and the pellets were
127 resuspended in 800 µL of lysis solution. Samples were incubated for 1 h at 60°C, 10 min at
128 95°C and kept at -20°C. When an inhibition of the PCR reaction was observed, DNA was re-
129 extracted with phenol/chloroform/isoamyl(ic) alcohol (25/24/1) (Marois et al., 2004). PCR
130 assays were carried out as previously described for *M. hyopneumoniae* (Calsamiglia et al.,
131 1999), *A. pleuropneumoniae* (Schaller et al., 2001), *Haemophilus parasuis* (Oliveira et al.,
132 2001) and *Streptococcus suis* (Marois et al., 2004). A new PCR test was developed for *P.*
133 *multocida* detection. PCR mixture contained PCR buffer (67 mM Tris-HCl, 16 mM (NH₄)₂SO₄,
134 0.01% Tween 20, 2.5 mM MgCl₂ [pH 8.8]), 500 µM of each deoxyribonucleoside triphosphate
135 (Eurobio, Les Ulis, France), 600 nM of Pm16S/3-f (AAGGGATGTTGTAAATAGATAGC)
136 and Pm16S/6-r (GCTTCGGGCACCAAGCATAT) primers, 1.5 units of *Taq* DNA polymerase
137 (Eurobio), and 5 µl of the DNA template. Amplification was performed in GeneAmp PCR
138 system 9700 (Applied Biosystem). The reaction procedure consisted of initial denaturation at
139 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s,
140 and extension at 72°C for 60 s and final elongation at 72°C for 10 min. The amplified product
141 (411 pb) was revealed in a 2% agarose gel in TBE buffer (90 mM Tris, 90 mM borate, 2.5
142 mM EDTA [pH 8]) for 1 h at a constant voltage of 125 V. The specificity of this PCR assay
143 was tested with a collection of 75 strains representing 33 toxigenic and non toxigenic *P.*
144 *multocida* subsp. *multocida* as well as seven *P. multocida* subsp. *septica* or *gallicida* and 35

145 other bacterial species isolated from the respiratory tract of pigs, among them *A.*
146 *pleuropneumoniae*, *H. parasuis*, *M. hyopneumoniae* and *S. suis*. The detection threshold
147 was 3 *P. multocida* CFU/assay.

148 Firstly, all the samples were analysed by PCR, then the positive samples were cultured. In
149 order to isolate *A. pleuropneumoniae*, *P. multocida*, *H. parasuis* and *S. suis*, 10 µL of each IS
150 were placed onto PPLO agar (Difco, Cergy Pontoise, France), supplemented with 10 µg/ml
151 of nicotinamide dinucleotide (β-NAD), 1 mg/ml of glucose and 5% of decomplexed horse
152 serum agar (Savoie et al., 2000). Media were incubated overnight at 37°C in 5% CO₂ and all
153 bacterial colonies were identified by PCR. *M. hyopneumoniae* was cultivated on modified
154 Friis medium (Marois et al., 2007).

155

156 2.5. Microscopic examinations

157 Microscopic lung examinations were conducted on each SPF pig. Lung samples (a piece of
158 the right middle lobe) were fixed in 10% buffered formalin: paraffin-embedded sections were
159 cut at 5 µm, stained by a trichrome coloration (hematoxylin, eosin and safran) and examined
160 by light microscopy.

161

162 3. Results

163 3.1. Post-mortem examinations of the lungs at slaughter

164 No macroscopic lung lesions were observed in the SPF pigs.

165 During the four-hour wait of SPF pigs of group 1, 3.828 finishing pigs were
166 slaughtered and post-mortem examinations of lungs were carried out on 160 randomly
167 selected pigs originating from 7 herds, slaughtered one hour before the SPF pigs.
168 Pneumonia was observed in 70 % of lungs and the mean of pneumonic scores was 3.30 (±
169 4.20). Recovering lesions (corresponding to early infections) were also noticed in 34 % of
170 lungs. Pleuritis and abscesses were observed in 5.6 % and 3.2 % of the pigs, respectively.

171 Post-mortem examinations were also carried out on 160 randomly selected lungs of
172 3.627 conventional finishing pigs from 8 farms, slaughtered one hour before the arrival of the
173 second group of SPF pigs (4 SPF pigs without any contact with conventional finishing pigs).

174 Pneumonia was observed in 80 % of lungs and the mean score was 5.10 (\pm 5.30). Thirteen
175 per cent of them had also recovering lesions. Pleuritis and abscesses were observed in 11.3
176 % and 1.3 % of the pigs, respectively.

177

178 3.2. Detection of pathogenic respiratory bacteria

179 3.2.1. Animals and lorry

180 Using culture and PCR tests, pathogenic respiratory bacteria were neither detected from the
181 SPF pigs nor from the lorry before departure for the slaughterhouse.

182 Results of the bacteriological investigations performed on the SPF pigs at the
183 slaughterhouse, are presented in Table 1. Major pathogenic respiratory bacteria (*M.*
184 *hyopneumoniae*, *A. pleuropneumoniae*, *P. multocida*, *H. parasuis*, and *S. suis*) were
185 detected in the SPF pigs by PCR, whatever the group of SPF pigs (group 1: with contact or
186 group 2: without any contact, with finishing pigs at slaughter). One to five bacterial species
187 were detected in each SPF pig. Except for *M. hyopneumoniae*, the percentages of positive
188 samples were higher in pigs of group 2. The total number of positive results, obtained with
189 tracheal swabs, was higher than with the other samples. Nevertheless, *M. hyopneumoniae*,
190 *A. pleuropneumoniae*, *H. parasuis* and *S. suis*, were also detected from nasal cavities and
191 palatine tonsils, particularly from the pigs of group 1. PCR assays were negative from the
192 right middle lobe of the lungs.

193 The culture of the microorganisms was fastidious, nevertheless, three and four
194 bacterial species were isolated in group 2 and in group 1, respectively. *H. parasuis* and *S.*
195 *suis* were the most frequent bacteria in the two groups. Except for *M. hyopneumoniae*, never
196 isolated from the respiratory tract of the SPF pigs, the most appropriate sites for bacteria
197 cultivation were nasal cavities, tonsils and trachea. Respiratory bacteria were not isolated
198 from the lungs.

199

200 3.2.2. Scalding tank water

201 During the collection of samples A (corresponding to group 1) and samples B (corresponding
202 to group 2), the average temperatures inside the scalding tank water were 61.1°C (\pm 0.4°C)
203 and 60.7°C (\pm 0.3°C), respectively. The pigs stayed in the water for about 7 min.
204 Bacteriological results are shown in Table 2. All bacterial species, followed during this study,
205 were detected in the scalding tank water by PCR tests. Before the onset of slaughter, *M.*
206 *hyopneumoniae*, *A. pleuropneumoniae* and *S. suis* were detected in five samples A, while
207 samples B were PCR negative at the same moment. The following samples (T+4 h and T+8
208 h) were PCR positive for the five bacterial species, except for *H. parasuis* at T+8 h.
209 *P. multocida* was isolated from samples B, in four cases (T+4 h and T+8 h).

210

211 3.3. Microscopic examinations

212 Microscopic lung examinations of the SPF pigs at the end of the slaughter process, revealed
213 severe alterations of the respiratory tract of 4/9 pigs of group 1 (Fig.1). In these pigs, an
214 exfoliation of the bronchial epithelial cells and a dilation of the capillary vessels were
215 observed (Fig.1 B). Moreover, the examinations showed cellular debris, scales and bacterial
216 accumulations in the bronchial lumen (Fig. 1 C and D). In 9/13 pigs, the structure of the
217 respiratory tract was normal (Fig. 1 A).

218

219 4- Discussion

220 In the conditions described in this study, major pathogenic respiratory bacteria (*M.*
221 *hyopneumoniae*, *A. pleuropneumoniae*, *P. multocida*, *H. parasuis* and *S. suis*) were detected
222 in the respiratory tract of SPF pigs slaughtered in a conventional slaughterhouse. It is widely
223 accepted that transportation of pigs, from farms to slaughterhouses, is a crucial risk factor of
224 cross-contaminations between pigs. In our study, transportation could not have any influence
225 on the transmission of respiratory pathogens to the SPF pigs.

226 The bacteriological results suggest that the SPF pigs became contaminated (1) by the
227 slaughterhouse environment or (2) by contact with infected finishing pigs during the lairage
228 period (pigs of group 1 only) or (3) by the scalding water.

229 A rapid contamination occurred in the two groups of SPF pigs in the slaughterhouse. PCR,
230 more sensitive than culture, was able to detect five bacterial species. Trachea was the most
231 appropriate site to detect these pathogens. Moreover, four and three species were isolated in
232 groups 1 and 2, respectively. One cause of contamination of SPF pigs was probably the
233 microbial flora present in the slaughterhouse environment. Previous studies indicated that *M.*
234 *hyopneumoniae* and *A. pleuropneumoniae* could be transmitted by aerosols over short
235 distances (Jobert et al., 2000; Otake et al., 2002; Gottschalk and Taylor, 2006; Thacker,
236 2006). The potential role of air for dissemination of pathogens is accepted, for instance, *M.*
237 *hyopneumoniae* was detected in air samples from pig herds (Stärk et al., 1998). Effective
238 control of respiratory pathogens depends on an optimal environment (air quality,
239 temperature, etc) but factors, such as high relative humidity, mixed infections and stress may
240 encourage the development and the spread of respiratory pathogens in pigs and in their
241 environment (Gottschalk and Taylor, 2006; Pijoan, 2006).

242 The main route of spread of respiratory pathogens is by direct contact from pig to pig
243 (Thacker, 2006). The time spent in lairage at the slaughterhouse (four hours in our study)
244 might be a risk factor for naïve pigs, as described for *Salmonella* (Beloeil et al., 2004). In
245 experimental conditions, *M. hyopneumoniae* was quickly transmitted to sentinel pigs and was
246 detected in nasal, tracheal and bronchial swabs (Meyns et al., 2004; Fano et al., 2005;
247 Marois et al., 2007). Similar results were described with *S. suis* and *A. pleuropneumoniae*.
248 (Jobert et al., 2000; Berthelot-Hérault et al., 2001, Marois et al., 2004) and with *P. multocida*
249 (de Jong, 2006). Nevertheless, our results showed that, except for *M. hyopneumoniae*,
250 percentages of PCR positive samples were higher in pigs of group 2. Percentages of positive
251 cultures were similar in the two groups, but *A. pleuropneumoniae* was not isolated from pigs
252 of group 2. These results suggest that a contact between the SPF pigs and the finishing pigs,
253 during 4 h in the slaughterhouse, had little or no influence on the transmission of respiratory
254 pathogens.

255 The scalding tank appeared to be a critical point of the slaughtering process. Before the
256 onset of slaughter, DNA from three major respiratory pathogens was detected in water of the
257 scalding tank (samples A). After the passage of finishing pigs in the scalding water (more

258 than 3.600 pigs in each case), and before the slaughtering of SPF pigs, the number of
259 positive samples of water increased and five bacterial species were recovered by PCR. Four
260 hours later, *H. parasuis* was not detected from the water samples. Post-mortem
261 examinations of 320 lungs of finishing pigs, before the slaughtering of SPF pigs, showed lung
262 lesions in a large proportion of them. No bacteriological analysis was carried out from these
263 lungs. Nevertheless, it is well known that the bacterial pathogens involved in respiratory
264 diseases of pigs are the species detected from pigs and scalding water in the present study.
265 Thus, the scalding tank water was contaminated by infected finishing pigs during the day of
266 slaughter. Interestingly, viable *P. multocida* were detected from four samples of water. In the
267 scalding tank water, *P. multocida* was probably surrounded by organic material and was
268 protected against a high temperature. According to de Jong, (2006), *P. multocida* can survive
269 10 min at 60°C. During the scalding, some water contaminated by respiratory pathogens, can
270 reach the trachea and the lungs of recently killed pigs. Histological results were in agreement
271 with this hypothesis. Contaminated water from the scalding tank water invaded bronchi of
272 some SPF pigs and bacterial accumulations were observed in the bronchial lumen.
273 To conclude, this experimental study showed that bacterial population present in
274 slaughterhouses might contribute to transmit pathogens between pigs. Nevertheless, the
275 scalding tank water was the critical point of the slaughtering process and an important source
276 of contamination for pigs. Checks routinely conducted at slaughter, for respiratory disorders
277 in finishing pigs, are very useful for veterinarians and have to be carried on, but nasal
278 cavities, tonsils and trachea are not appropriate for bacteriological investigations. Despite we
279 could not detect pathogenic respiratory bacteria from the lungs of the SPF pigs,
280 bacteriological investigations at slaughter have to be used with carefulness.

281

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286

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362

363 Table 1: Detection of pathogenic respiratory bacteria from 13 SPF pigs, at the end of the
364 slaughter process.

365 NS: nasal swabs; TOS: tonsillar swabs; WT: whole tonsils; TS: tracheal swabs; LS: lung
366 swabs

367 *4 h before slaughtering

368 Mhp: *Mycoplasma hyopneumoniae*, App: *Actinobacillus pleuropneumoniae*, Pm: *Pasteurella*
369 *multocida*, Hps: *Haemophilus parasuis*, S. suis : *Streptococcus suis*.

370

371

372 Table 2: Detection of pathogenic respiratory bacteria from the scalding tank water (water
373 samples taken thrice (T0, T+4, T+8) at 5 different places in the tank: A (9 SPF pigs), B (4
374 SPF pigs).

375 Mhp: *Mycoplasma hyopneumoniae*, App: *Actinobacillus pleuropneumoniae*, Pm: *Pasteurella*
376 *multocida*, Hps: *Haemophilus parasuis*, S. suis : *Streptococcus suis*.

377

378

379 Figure 1: Microscopic examinations in the lungs of three SPF pigs (group 1) checked at the
380 end of the slaughter process.

381 A: Normal structure of the bronchial epithelium (x200)

382 B: Exfoliation of the bronchial epithelial cells, dilated capillary vessels (x200)

383 C: Cellular debris, scales and bacterial accumulations in the bronchial lumen (x25)

384 D: Cellular debris, scales and bacterial accumulations in the bronchial lumen (x100).

385

386

386

387 Table 1: Detection of pathogenic respiratory bacteria from 13 SPF pigs, at the end of the
 388 slaughter process.

389

Groups of SPF pigs	Samples from SPF pigs	Positive PCR					Positive cultures				
		Mhp	App	Pm	Hps	S. suis	Mhp	App	Pm	Hps	S. suis
1 (n=9) waiting in lairage *	NS	3	1	0	3	7	0	0	0	3	7
	TOS	2	1	0	8	3	0	0	0	8	2
	WT	1	4	3	0	0	0	0	1	0	0
	TS	7	7	1	6	3	0	2	1	5	2
	LS	0	0	0	0	0	0	0	0	0	0
	Total (n=45)	13	13	4	17	13	0	2	2	16	11
		28.9%	28.9%	8.9%	37.8%	28.9%	0%	4.4%	4.4%	35.6%	24.4%
2 (n=4)	NS	1	2	1	3	3	0	0	0	2	3
	TOS	0	0	1	3	2	0	0	1	2	1
	WT	0	1	1	1	1	0	0	0	1	1
	TS	2	3	4	3	4	0	0	0	2	4
	LS	0	0	0	0	0	0	0	0	0	0
	Total (n=20)	3	6	7	10	10	0	0	1	7	9
		15%	30%	35%	50%	50%	0%	0%	5%	35%	45%

390

391 NS: nasal swabs; TOS: tonsillar swabs; WT: whole tonsils; TS: tracheal swabs; LS: lung
 392 swabs

393 *4 h before slaughtering

394 Mhp: *Mycoplasma hyopneumoniae*, App: *Actinobacillus pleuropneumoniae*, Pm: *Pasteurella*
 395 *multocida*, Hps: *Haemophilus parasuis*, S. suis : *Streptococcus suis*.

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398 Table 2: Detection of pathogenic respiratory bacteria from the scalding tank water (water
399 samples taken thrice (T0, T+4, T+8) at 5 different places in the tank: A (9 SPF pigs), B (4
400 SPF pigs).

401

Samples of water	Time (h)	Positive PCR					Positive cultures				
		Mhp	App	Pm	Hps	S. suis	Mhp	App	Pm	Hps	S. suis
A	T0	2	2	0	0	1	0	0	0	0	0
	T+4	5	4	5	3	1	0	0	0	0	0
	T+8	5	5	2	0	1	0	0	0	0	0
Total (n=15)		12	11	7	3	3	0	0	0	0	0
B	T0	0	0	0	0	0	0	0	0	0	0
	T+4	5	5	5	2	5	0	0	3	0	0
	T+8	5	2	5	0	4	0	0	1	0	0
Total (n=15)		10	7	10	2	9	0	0	4	0	0

402

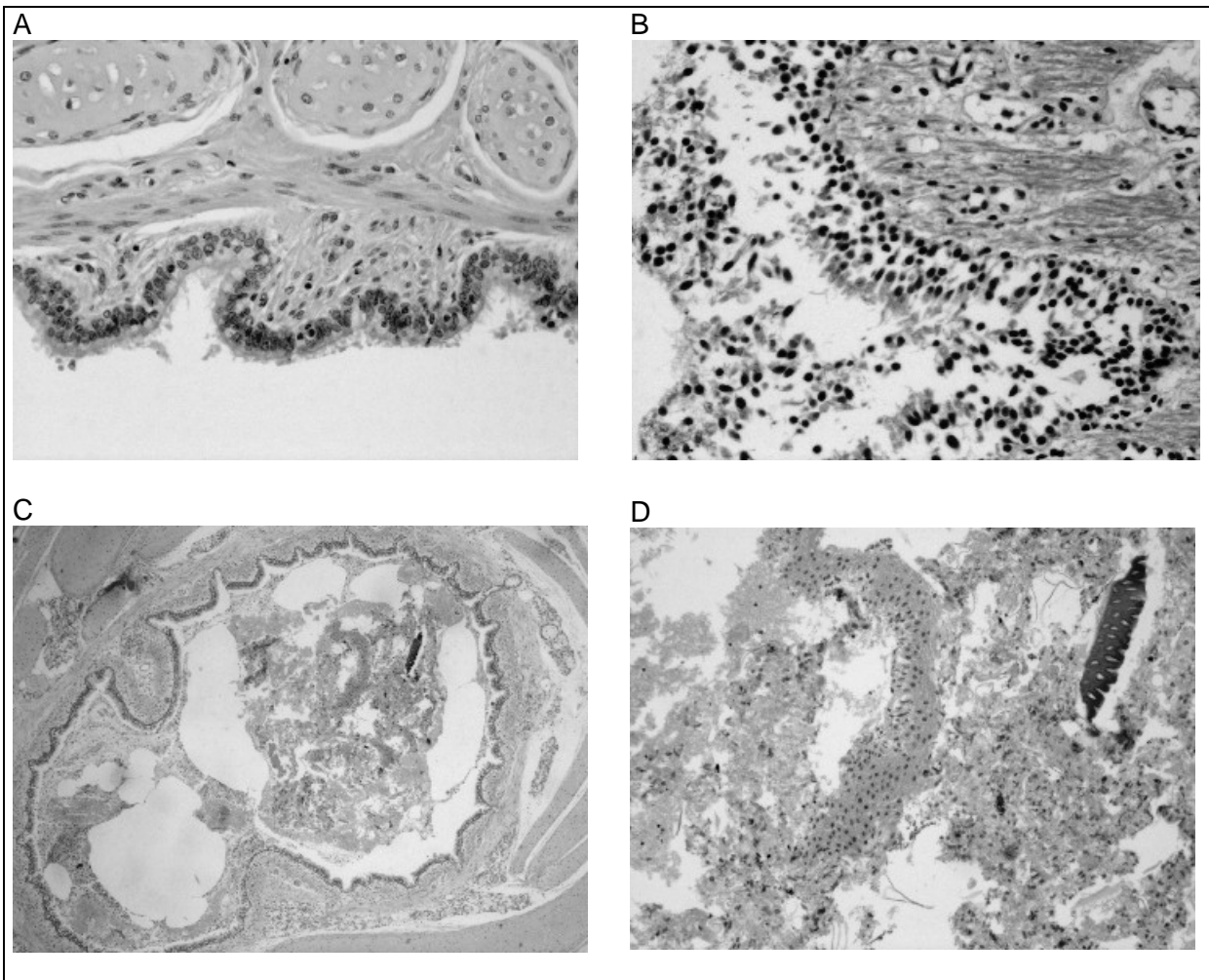
403 Mhp: *Mycoplasma hyopneumoniae*, App: *Actinobacillus pleuropneumoniae*, Pm: *Pasteurella*

404 *multocida*, Hps: *Haemophilus parasuis*, S. suis : *Streptococcus suis*.

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410 Figure 1: Microscopic examinations in the lungs of three SPF pigs (group 1) checked at the
411 end of the slaughter process.

412 A: Normal structure of the bronchial epithelium (x200)

413 B: Exfoliation of the bronchial epithelial cells, dilated capillary vessels (x200)

414 C: Cellular debris, scales and bacterial accumulations in the bronchial lumen (x25)

415 D: Cellular debris, scales and bacterial accumulations in the bronchial lumen (x100).

416