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Title: Estimation of the sensitivity of four sampling methods for *Mycoplasma hyopneumoniae* detection in live pigs using a Bayesian approach

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31 swabbing. Swabbing the nasal cavities appeared to be the least sensitive method. Significantly higher
32 amounts of *M. hyopneumoniae* DNA were found at the sites of tracheo-bronchial sampling than in the
33 nasal cavities or at the oral-pharyngeal site ($p < 0.001$). There was no difference between the tracheo-
34 bronchial washing and the tracheo-bronchial swabbing results ($p > 0.05$). Our study indicated that
35 tracheo-bronchial swabbing associated with Real-Time PCR could be an accurate diagnostic tool for
36 assessing infection dynamics in pig herds.

37

38 Keywords: *Mycoplasma hyopneumoniae*, sampling methods, real time PCR, bayesian analysis

39

40 1. Introduction

41 *Mycoplasma hyopneumoniae* is the primary aetiological agent of enzootic pneumonia in pigs, a
42 chronic respiratory disease of worldwide distribution (Thacker, 2006). *M. hyopneumoniae*, in
43 association with bacteria and viruses of the respiratory tract, is also involved in the pathogenesis of
44 Porcine Respiratory Disease Complex (PRDC) (Sibila et al., 2009). Monitoring *M. hyopneumoniae*
45 contamination in live pigs provides useful information on the dynamics of infection within a herd
46 together with insight into the factors influencing the infection pattern and the design of suitably timed
47 preventive and/or control strategies. These investigations rely on the availability of accurate and
48 reliable sampling sites and laboratory analyses. Although the detection of *M. hyopneumoniae* by
49 bacteriological culture is considered as the “gold standard”, difficulties in culturing this micro-organism
50 have led to the development of other assays, especially PCR based technology (Thacker, 2006; Sibila
51 et al., 2009). The PCRs currently performed on samples from live pigs have some limitations since
52 they only provide qualitative results. Little is known about the bacterial load carried by the animals and
53 whether this differs in different parts of the respiratory tract. This information is important when
54 assessing (i) the potential of different sampling techniques to detect contaminated animals and (ii) the
55 ability of these animals to shed bacteria as high levels are more likely to result in more rapid
56 spreading. Recently, a quantitative real time PCR assay was developed and validated on samples
57 taken from experimentally infected pigs (Marois et al., in press).

58 Different sampling sites and types were used to detect *M. hyopneumoniae* infection by PCR
59 techniques on live pigs subjected to experimental and natural challenges *i.e.* nasal, tonsillar, tracheal
60 swabs or brushes and tracheo-bronchial and broncho-alveolar washings (Baumeister et al., 1998;

61 Calsamiglia et al., 1999a; Calsamiglia et al., 1999b; Verdin et al., 2000b; Kurth et al., 2002; Otagiri et
62 al., 2005; Moorkamp et al., 2008; Moorkamp et al., 2009). For practical reasons, the nasal cavities are
63 the most frequently chosen sampling sites to assess *M. hyopneumoniae* contamination of live pigs,
64 under field conditions. However, in experimental trials, tracheal and tracheo-bronchial washings are
65 the most efficient samples for detecting *M. hyopneumoniae* (Kurth et al., 2002; Marois et al., 2007).
66 The aims of this study were therefore (1) to compare 4 sampling techniques: nasal swabbing, oral-
67 pharyngeal brushing, tracheo-bronchial swabbing and tracheo-bronchial washing for the detection of
68 *M. hyopneumoniae* in naturally infected live pigs and (2) to validate the use of a quantitative real-time
69 PCR to assess the amount of *M. hyopneumoniae* in samples taken at different levels of the airways.

70

71 **2. Material and methods**

72 **2.1. Animals and study design**

73 **2.1.1. Herd selection**

74 The study was carried out on a two-sites farrow-to-finish herd located in Brittany (France), managed
75 all-in all-out by room with a 3-week batch interval. According to the farm's veterinarian, the herd was
76 chronically affected by respiratory disorders. Coughing was typically expressed during the finishing
77 phase and respiratory disorders were the main reason for medication. Pigs were vaccinated against
78 *M. hyopneumoniae* at 4 and 7 weeks of age. Pneumonia was regularly observed at the
79 slaughterhouse. The farm was visited before beginning the experiment, to confirm the clinical signs
80 and *M. hyopneumoniae* infection of a batch of 180 days old finishing pigs. Tracheo-bronchial swabs
81 from a sample of 10 randomly selected pigs gave 6 positive results in a nested-PCR detection of *M.*
82 *hyopneumoniae* DNA (as detailed below). The study was carried out on a subsequent batch of 87
83 finishing pigs, 3 weeks after the first check visit.

84

85 **2.1.2. Animals and sampling scheme**

86 The pigs were housed in a mechanically ventilated finishing room, containing eight pens. No treatment
87 was administered during the three weeks before the study. Sample size calculations were based on
88 expected prevalence and sensitivity parameters by applying the method for diagnostic accuracy of two
89 paired tests described by Zhou et al., (2002). Data from an experimental study were used to produce
90 hypotheses on nasal swabbing, tracheo-bronchial swabbing and tracheo-bronchial washing

91 sensitivities and correlations (Marois et al., 2007). The expected prevalence, based on the first visit,
92 was 55 %. Sample size calculations were based on a significance level of 5 per cent and a power of
93 75 %. A minimum sample size of 61 pigs was required to estimate a difference in sensitivity of 0.4
94 between nasal swabbing and tracheo-bronchial swabbing and between nasal swabbing and oral-
95 pharyngeal brushing with a correlation coefficient of 0.3. A minimum sample size of 55 pigs was
96 needed to detect a suspected difference in sensitivity of 0.35 between tracheo-bronchial swabbing and
97 tracheo-bronchial washing with a correlation coefficient of 0.8. A minimal sample size of 54 pigs was
98 required to detect a difference in sensitivity of 0.35 between oral-pharyngeal brushing and tracheo-
99 bronchial swabbing and between oral-pharyngeal brushing and tracheo-bronchial washing, with a
100 correlation coefficient of 0.7. A sample was constituted of 60 pigs randomly selected from the 8 pens.

101 **2.1.3. Sampling techniques in live pigs**

102 2.1.3.1. Preliminary assessment under experimental conditions

103 Before their use in live pigs under field conditions, the feasibility and the characteristics of the four
104 sampling techniques were tested in preliminary experimental studies, in both specific-pathogen-free
105 (SPF) and *M. hyopneumoniae* experimentally infected SPF pigs, which served as negative and
106 positive control groups respectively (Marois et al., 2007; Marois et al., in press). For the positive
107 control group, data on day 21 post-inoculation of pigs intratracheally infected with 10^9 UCC of *M.*
108 *hyopneumoniae* strain were considered. Results obtained from isolation of *M. hyopneumoniae* by
109 bacteriological culture in the sampling specimens were used in the positive control group to test the
110 ability of the four sampling techniques to detect viable *M. hyopneumoniae* in each sampling site. For
111 the negative control group, results from both culturing and PCR technique were considered to assess
112 the probability of false positive results from each sampling technique in negative SPF pigs.

113

114 2.1.3.2. Sample collection under field conditions

115 The animals were restrained with a conventional cable snare over the maxilla. Each pig was subjected
116 to 4 samplings, performed by previously trained technicians, in the following order: oral-pharyngeal
117 brushing, tracheo-bronchial swabbing, tracheo-bronchial washing and nasal swabbing. The pig's
118 mouth was held open with a gag to obtain the oral-pharyngeal and tracheo-bronchial samples. Oral-
119 pharyngeal samples were obtained by swabbing the surface of the oral-pharyngeal cavity thoroughly
120 but gently with a brush protected by a catheter (Ori Endometrial Brush™, Orifice Medical AB, Ystad,

121 Sweden). Tracheo-bronchial swabs were collected with a sterile catheter used for tracheal intubations
122 (Euromedis, Neuilly-sous-Clermont, France). The catheter was deeply inserted into the trachea as the
123 pig inspired, then rotated and moved up-and-down. Tracheo-bronchial washing samples were
124 collected by transtracheal aspiration: 10 ml of 0.1 M PBS pH 7.4 containing 0.15 M NaCl were
125 introduced into the trachea as deeply as possible with a sterile catheter and immediately aspirated.
126 For nasal sampling, both nasal cavities were swabbed with "CytoBrushs" (VWR International,
127 Fontenay-sous-Bois, France), inserted into the nostrils by rotation to reach deeply into the turbinates.
128 All samples, except the tracheo-bronchial washing fluid, were placed in 2 ml of Buffered Peptone
129 Water Broth. They were individually identified and delivered to the laboratory for processing on the day
130 of collection (Initial Suspension: IS).

131

132 **2.2. Laboratory analyses**

133 **2.2.1. DNA preparation**

134 Samples were prepared for PCR assays as described by Kellog and Kwok (1990) (Kellog and Kwok,
135 1990). Briefly, 1 ml of each IS was centrifuged (12.000×g, 4°C, 20 min) and the pellets were
136 resuspended in 800 µl of lysis solution. Lysates were incubated for 1 h at 60°C, 10 min at 95°C and
137 then kept at -20°C.

138 **2.2.2. Nested-PCR**

139 *M. hyopneumoniae* DNA was identified by modified nested-PCR (Calsamiglia et al., 1999b). The PCR
140 mixture contained PCR buffer (67 mmol/l Tris-HCl, 16 mmol/l (NH₄)₂SO₄, 0.01% Tween 20, 1.5 mmol/l
141 MgCl₂ [pH 8.8]), 200 µmol/l of each deoxyribonucleoside triphosphate (Eurobio, Les Ulis, France), 200
142 nmol/l of each primer, 1 unit of *Taq* DNA polymerase (Eurobio), and 5 µl of the DNA template.
143 Samples were classified as nested-PCR positive or nested-PCR negative.

144 **2.2.3 Real-Time PCR**

145 The RT-PCR assay developed by Marois et al., (in press) was used to assess the amount of *M.*
146 *hyopneumoniae* DNA in each sample. The RT-PCR target defined in the *p102* gene was used in this
147 assay.

148 Briefly, the mixture contained iQsupermix (20 mmol/l Tris-HCl, 50 mmol/l KCl, 3 mmol/l MgCl₂ [pH 8.4],
149 800 µmol/l of each deoxyribonucleoside triphosphate, 0.625 units *Taq* polymerase and stabilizers)
150 (Bio-Rad, Marnes-La-Coquette, France), 500 nmol/l of each primer, 300 nmol/l of each probe and 5 µl

151 of the DNA template. In the negative control, the DNA template was replaced with double-distilled
152 water. Amplification was performed with the Chromo4 real-time PCR Detection System (Bio-Rad). The
153 reaction procedure consisted of denaturation at 95°C for 3 min then 40 cycles of denaturation at 95°C
154 for 15 s and annealing/extension at 60°C for 60 s.

155

156 **2.3. Statistical analysis**

157 **2.3.1. Estimation of the characteristics (sensitivity and specificity) of the sampling methods**

158 **without a gold standard**

159 2.3.1.1. Model description

160 A Bayesian analysis framework described by Berkvens et al., (2006) was applied to estimate the
161 characteristics of four conditionally dependent tests in a single population and without a gold standard.
162 Results obtained with the nested-PCR were used. A model, based on a multinomial distribution and
163 including all possible interactions between the four individual tests requires the estimation of 31
164 parameters. These are the prevalence, sensitivity and specificity of the first test, two conditional
165 sensitivities and two conditional specificities for the second test, four conditional sensitivities and four
166 conditional specificities for the third test, and finally eight conditional sensitivities and eight conditional
167 specificities for the fourth test. The parameters are defined in Table 1. Such a model is inestimable
168 because the data (16 'classes' of test results) provide only 15 degrees of freedom. The model building
169 strategy consisted of incorporating extraneous prior information to reduce the number of parameters to
170 be estimated and the range of values for a specific parameter (Dunson, 2001). For some parameters,
171 no objective prior information could be formulated and it was therefore necessary to leave the prior
172 information on these parameters non informative (Gelman et al., 2002).

173

174 2.3.1.2. Prior information

175 Beta distributions $Be(a,b)$ were used as priors for the parameters required in the model. Parameters
176 distributions were determined on the basis of previous external data. The prior information for the
177 proportion of nested-PCR positive pigs was based on the laboratory results from the first visit to the
178 farm and from previous field studies which indicated that, at the end of the finishing phase, the level of
179 nested-PCR positive pigs would be high, whatever the sampling method used (Calsamiglia et al.,
180 1999a; Verdin et al., 2000b). Since samples taken from SPF pigs gave negative results, a

181 deterministic constraint was used for the specificities of all four sampling methods which was taken as
182 equal to one (Marois et al., 2007; Marois et al., in press). These assumptions led to the exclusion of all
183 parameters pertaining to false positive results and in turn to the reduction of parameters that had to be
184 estimated in the model. The prior distributions of conditional probabilities were based on previous
185 experimental data (Kurth et al., 2002; Otagiri et al., 2005; Marois et al., 2007). Since no reliable prior
186 information was available for the tracheo-bronchial swabbing parameters (Table 1: p16 to p31), vague
187 priors were used. The sensitivity of the parameter estimation to the choice of priors was assessed by
188 comparing 3 models incorporating different sets of prior distributions ranging from vague priors (M1) to
189 more informative ones (M3) (Table 2).

190 2.3.1.3. Model implementation

191 The models were run using the freeware program WinBUGS (Spiegelhalter et al., 1996). Parameter
192 estimates were based on analytical summaries of 25,000 iterations of the Gibbs sampler with a burn-in
193 phase of 5,000 iterations. Three parallel chains were run with different starting values randomly
194 chosen from uniform distributions (0,1).

195 2.3.1.4. Model assessment

196 Model convergence was assessed using the Raftery and Lewis tests for single chains and the
197 Gelman-Rubin diagnosis for the 3 parallel chains. The models were compared on the basis of the
198 deviance information criterion (DIC), the number of parameters estimated in the model (p_D) and of the
199 Bayesian p-value (Bayes-p).

200

201 **2.3.2. Real Time-PCR results**

202 RT-PCR data were not normally distributed (Kolmogorov Smirnov, $p < 0.05$) and showed an excess in
203 zeros. Differences between two sampling methods were therefore compared by a two-part model
204 adapted to paired data (Lachenbruch, 2001). The two-part model was based on a Mc Nemar chi-
205 square test to assess for differences in zero proportions and a conditional Wilcoxon test to test for
206 differences in the non-zero continuous part of the data. All comparisons were performed using the free
207 software R (R Development Core Team, 2008).

208

209 **3. Results**

210 **3.1. Preliminary experimental studies**

211 Under experimental conditions, all sampling methods gave negative result by PCR or by
212 bacteriological culture in SPF pigs (Table 3). In the positive control group, *i.e.* experimentally infected
213 pigs, *M. hyopneumoniae* was isolated from 13% of the nasal cavities and 65% of the oro-pharyngeal
214 area. The micro-organism was also recovered from 70% and 67% of the tracheo-bronchial swabbing
215 and tracheo-bronchial washing respectively (Table 3).

216

217 **3.2. *Mycoplasma hyopneumoniae* detection in field conditions**

218 The cross classified results of the four sampling methods for the detection of *M. hyopneumoniae* are
219 shown in Table 4. Eight (13.3 %) of the 60 pigs sampled for *M. hyopneumoniae* detection by nested-
220 PCR, were considered positive by nasal swabbing, 24 (40.0%) tested positive by oral-pharyngeal
221 brushes, 32 (53.3 %) were positive by tracheo-bronchial washing and 36 (60.0%) were positive by
222 tracheo-bronchial swabbing. Out of the 8 nested-PCR positive pigs by nasal swabbing, 7 were also
223 positive by the 3 other sampling methods. In total, 70% of the sampled pigs were positive by nested-
224 PCR for at least one sampling method.

225 **3.3. Bayesian estimation of the characteristics of the sampling methods**

226 Using prior knowledge of test specificity (equal to one for all sampling methods) the number of
227 parameters to be estimated in the conditionally dependent test model could be reduced to seventeen.
228 The model could be slightly improved, in comparison to M1 for which all priors were non informative,
229 by including information about the proportion of *M. hyopneumoniae* positive pigs, which was assumed
230 to be >0.5 with a mode at 0.7 with 95 % certainty, slightly improve the model in comparison with M1
231 for which all priors were non informative (Table 5). The Bayes-p was 0.51, the DIC was 47.4 and p_D
232 was 7.7. Additional constraints did not greatly improve the model. The mean estimated sensitivity and
233 specificity along with the 95 % credibility interval of each test and for each model constructed are
234 presented in Table 6. Whatever the model, nasal swabbing had the lowest sensitivity and tracheo-
235 bronchial swabbing the highest with mean sensitivities of 19 % and 74 %, respectively.

236

237 **3.4. Quantitative evaluation of *M. hyopneumoniae***

238 *M. hyopneumoniae* DNA was amplified by RT-PCR assay in 41 of the 60 tested pigs (68.3 %). All pigs
239 shown to be positive by RT-PCR assay were also positive by nested-PCR. The amounts of *M.*
240 *hyopneumoniae* DNA detected in samples ranged from 0 to 1.4×10^8 fg/ml whatever the sampling

241 method. The distribution of the amounts of *M. hyopneumoniae* DNA according to the sampling method
242 is given in Figure 1. The highest amounts of *M. hyopneumoniae* DNA assessed in samples from nasal
243 swabs, oral-pharyngeal brushing, tracheo-bronchial washing and tracheo-bronchial swabbing were
244 2.5×10^4 fg/ml, 1.4×10^6 fg/ml, 1.3×10^8 fg/ml and 1.4×10^8 fg/ml respectively. At least 1.0×10^7 fg/ml *M.*
245 *hyopneumoniae* DNA was found in one or more samples from 9 pigs (15%). The mean quantities of *M.*
246 *hyopneumoniae* DNA detected in live pigs by nasal swabbing, oral-pharyngeal brushing, tracheo-
247 bronchial washing and tracheo-bronchial swabbing were 7.0×10^2 fg/ml (SD= 3.5×10^3), 7.5×10^4 fg/ml
248 (SD= 2.3×10^5), 4.0×10^6 fg/ml (SD= 1.7×10^7) and 5.0×10^6 fg/ml (SD= 1.9×10^7), respectively. Significantly
249 higher amounts of *M. hyopneumoniae* DNA were detected at the tracheo-bronchial sampling sites than
250 in the nasal cavities or at the oral-pharyngeal site ($p < 0.001$). However, no differences were observed
251 between the results obtained for tracheo-bronchial washing and tracheo-bronchial swabbing ($p > 0.05$).
252 The mean amount of *M. hyopneumoniae* DNA recovered from nasal swabs was significantly lower
253 than the amount detected with the other sampling methods ($p < 0.001$).

254 4. Discussion

255
256 Since *M. hyopneumoniae* plays a crucial role in the Porcine Respiratory Complex, the availability of
257 accurate, rapid and easy to perform diagnostic tools is necessary for epidemiological and control
258 purposes. In field studies, *M. hyopneumoniae* infection in live pigs is often assessed by PCR
259 techniques performed on nasal swabs. However, experimental trials indicate that the optimal sampling
260 sites are located in the lower parts of the airways, namely the trachea and bronchi (Kurth et al., 2002;
261 Marois et al., 2007). Therefore the aim of our study was to assess the abilities of four sampling
262 methods to detect *M. hyopneumoniae* by nested-PCR on live pigs in a field context. Since the infection
263 status of the pigs tested under these conditions was unknown, and no “perfect test” (i.e. gold standard)
264 is available, the sensitivities of the sampling methods were analyzed using a Bayesian approach. To
265 the best of our knowledge, this is the first field study to use such an approach to evaluate four different
266 sampling methods for assessing *M. hyopneumoniae* infection in live pigs. Bayesian methodology has
267 already proved its usefulness in estimating the characteristics of diagnostic tests without a gold
268 standard when at least 3 tests are used to diagnose parasite infection in dogs, calves or pigs
269 (Berkvens et al., 2006; Geurden et al., 2006; Geurden et al., 2008). With Bayesian rather than
270 frequentist statistics, the uncertainty about all parameters is modeled with a probability that reflects the

271 scientific uncertainty of the unknown quantities (Joseph et al., 1995). The Bayesian approach allows
272 estimation of the test characteristics, based on cross-classified test results and prior information,
273 without the need for a well-defined and evaluated gold standard reference test. The choice of priors is
274 therefore a key point in such an approach. Prior information allows the number of parameters to be
275 estimated, in hierarchical conditionally dependent models, to be reduced (Berkvens et al., 2006).
276 Although these priors influence the model to a certain extent, estimated sensitivities can be obtained
277 by combining the collected data with external data from previously published studies (Calsamiglia et
278 al., 1999a; Verdin et al., 2000b; Kurth et al., 2002; Otagiri et al., 2005; Marois et al., 2007; Marois et
279 al., in press). Besides, the conclusions regarding the comparison of sensitivity of detection according
280 to sampling sites were not modified when more informative priors were included for several of the
281 parameters. In the present study the specificity of all sampling methods was fixed at one since the
282 nested-PCR assay was originally reported to be highly specific for *M. hyopneumoniae* as no
283 amplification of DNA from other mycoplasma species, or other micro-organisms commonly found in
284 pigs, was observed (Calsamiglia et al., 1999b). Furthermore, results from the preliminary experimental
285 trials indicate that none of the four sampling methods led to a positive reaction with any of the samples
286 from the negative control animals. In the present study, nasal swabbing was found to be the least
287 sensitive sampling method for detecting *M. hyopneumoniae* in live pigs under field conditions.
288 According to nested-PCR, the highest sensitivities for *M. hyopneumoniae* detection were obtained with
289 tracheo-bronchial washing and tracheo-bronchial swabbing, that of tracheo-bronchial swabbing being
290 slightly higher. Interestingly, oral-pharyngeal brushing was slightly more sensitive than nasal swabbing
291 but less able to detect infected animals than sampling at tracheo-bronchial sites. This seems to
292 indicate a gradual trend in *M. hyopneumoniae* detection along the respiratory tract, the likelihood of
293 detecting *M. hyopneumoniae* in infected pigs increasing with the depth, within the respiratory tract, of
294 the biological sample. This is in agreement with results obtained in experimental challenge studies to
295 compare different sampling sites for the detection of *M. hyopneumoniae* in infected live pigs (Kurth et
296 al., 2002; Marois et al., 2007; Marois et al., in press). Under field conditions, Verdin et al., (2000b)
297 showed that only one of 10 pigs, that tested positive by nested-PCR assay of tracheo-bronchial
298 washings, was detected positive by nasal swabbing. This better recovery of *M. hyopneumoniae* from
299 tracheo-bronchial sites, than from upper parts of the respiratory tract, is consistent with the fact that *M.*
300 *hyopneumoniae* binds to the ciliated epithelia of the airways and multiplies in the trachea and bronchi

301 (Blanchard et al., 1992; Kurth et al., 2002; Marois et al., 2007). During the first stages of the infection
302 process, colonization of the airways by the micro-organism results in loss of cilia and ciliated cells
303 (Underdahl et al., 1980; Blanchard et al., 1992). One may suppose that the sensitivity of sampling
304 method involving trachea would be greater with a higher proportion of ciliated epithelial cells, i.e. in the
305 early stage of infection. Moorkamp et al., (2008) showed that even if BALF is an appropriate material
306 for detecting the early stages of *M. hyopneumoniae* infection, it failed to recover some positive animals
307 in the case of chronic infection. The sensitivity of the sampling method is therefore suspected to vary
308 over the time of infection. In the present study carried out under field conditions, the exact stage of the
309 infection process of each naturally infected pig is unknown, some pigs being recently infected, i.e. in
310 the early stage of infection, and others being in a more chronic one. The ability of the sampling
311 methods to detect *M. hyopneumoniae* at various stages of infection could therefore not be tested,
312 which constitute a limitation of such a field study. Assessment of the effect of the stage of infection on
313 the recovery of *M. hyopneumoniae* in different sampling site need to be properly considered in further
314 experimental studies. Since Marois et al., (2007) suggested that several tracheo-bronchial washings
315 performed on the same pig could affect the extent of lung lesions, tracheo-bronchial swabs should
316 preferably be obtained from live pigs by using a sterile catheter in order to avoid effects on
317 slaughterhouse assessments of the lung lesion score. Nevertheless, as no test is perfect, sampling at
318 several sites should be recommended to increase sensitivity. Even if *M. hyopneumoniae* does not
319 normally colonize the nasal cavities, the organism may be detected at this site (Goodwin, 1972).
320 Hence, nostril swabbing is often used to assess the infected status of pigs under natural and
321 experimental conditions. However, nasal shedding is reported to be intermittent, as *M.*
322 *hyopneumoniae* is only detected in the nasal cavities for a limited period (Mattsson et al., 1995;
323 Calsamiglia et al., 1999b). This may partly explain the relative inaccuracy of this sampling method.
324 Another explanation could be that only a few *M. hyopneumoniae* cells were present in the upper
325 respiratory tract. Otagiri et al., (2005) showed that the *M. hyopneumoniae* titres in nasal swabs
326 obtained from experimentally infected pigs were generally one-hundred-fold less than those in lung
327 homogenates. Marois et al. (in press), using a quantitative Real-Time PCR, reported higher numbers
328 of *M. hyopneumoniae* cells in the trachea of experimentally infected pigs than in the nasal cavities and
329 tonsils. The mean quantity of *M. hyopneumoniae* was ten to ten-thousand times higher in the trachea
330 than in the nasal cavities, depending on the infective dose and time after inoculation. Furthermore,

331 similar quantities of the organism were obtained from trachea and lung samples. In the present study,
332 the mean quantity of *M. hyopneumoniae* DNA was 7×10^2 fg/ml, with 7.5×10^4 fg/ml obtained by nasal
333 and oral-pharyngeal sampling, and 10^6 fg/ml by tracheo-bronchial sampling. Data dealing with the
334 quantification of *M. hyopneumoniae* under field conditions are scarce. Verdin et al. (2000a), who
335 estimated the number of Mycoplasma cells in tracheo-bronchial washings of 8 finishing pigs found
336 titres ranging from 10^4 to 10^8 *M. hyopneumoniae* cells per millilitre of sample. Therefore our study is
337 the first to estimate the number of *M. hyopneumoniae* present in various clinical samples of the
338 airways of naturally infected pigs using a quantitative Real-Time PCR assay. It can be seen that the
339 gradual increase in sensitivity of *M. hyopneumoniae* detection of the four sampling methods, from
340 nasal swabs to tracheo-bronchial swabbing, as determined by nested-PCR assay, fitted well with the
341 progressive increase in number of *M. hyopneumoniae* along the respiratory tract of infected pigs, as
342 assessed by Real-Time PCR assay. The results also showed that within a batch of pigs, while some
343 pigs were either not contaminated with *M. hyopneumoniae* or only at low levels, other pigs shed high
344 numbers of mycoplasma in their airways. Up to 10^8 fg/ml of *M. hyopneumoniae* DNA was detected in
345 the lower part of the respiratory tract of the pigs. Although no distinction between dead and live
346 bacteria could be made by PCR assay, positive nested-PCR pigs were found to be infectious (Pieters
347 et al., 2009). Furthermore, results from the preliminary trials indicate that *M. hyopneumoniae* could be
348 isolated from samples collected in the three sampling sites in positive control pigs. Viable organisms
349 may therefore be present in the different parts of the airways, from nasal cavities to tracheo-bronchial
350 area with a better recovery rate in the lower parts of the respiratory tract. Therefore we can speculate
351 that pigs with a high *M. hyopneumoniae* DNA load are likely to infect susceptible pigs, by direct
352 contact or contaminated droplets during coughing, sneezing or breathing, which in turn contribute to
353 the persistence of infection. In PRDC, *M. hyopneumoniae* interact with other respiratory pathogens
354 such as bacteria and viruses (Sibila et al., 2009). In this context, one may wonder whether the
355 presence of such pathogens may influence the amount of *M. hyopneumoniae* in the airways. In the
356 present study, the farm was known to be regularly infected by Swine Influenza Viruses. However, the
357 individual status of the sampled pigs towards respiratory pathogens was not tested and remained
358 unknown. Assessment of the effect of co-infection on the quantities of *M. hyopneumoniae* in the
359 different parts of the respiratory tract would need to be investigated in further studies.

360 In conclusion, the results of the present study indicate that tracheo-bronchial swabbing is the most
361 sensitive sampling method for detecting *M. hyopneumoniae* in naturally infected live pigs, using nested
362 or Real-Time PCR assays. As far as practical aspects are concerned, swabbing the tracheo-bronchial
363 area with a sterile catheter is almost as convenient as obtaining nasal swabs under field conditions
364 and only requires adding a gag to the sampling equipment. Tracheo-bronchial swabbing ensures a
365 gain in diagnostic accuracy, being 3.5 times more sensitive than the nasal swabs commonly used in
366 pig farms. In combination with the RT-PCR assay it should provide a very useful method for
367 documenting the course of natural *M. hyopneumoniae* infections and studying the dynamics of
368 infection at both pig and herd levels.

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1 Table 1: Conditional probabilities for a four dependent tests model

2

p1*	$P(D^+)^{**}$
p2	$P(T_1^+ D^+)$
p3	$P(T_1^- D^-)$
p4	$P(T_2^+ D^+ \cap T_1^+)$
p5	$P(T_2^+ D^+ \cap T_1^-)$
p6	$P(T_2^- D^- \cap T_1^-)$
p7	$P(T_2^- D^- \cap T_1^+)$
p8	$P(T_3^+ D^+ \cap T_1^+ \cap T_2^+)$
p9	$P(T_3^+ D^+ \cap T_1^+ \cap T_2^-)$
p10	$P(T_3^+ D^+ \cap T_1^- \cap T_2^+)$
p11	$P(T_3^+ D^+ \cap T_1^- \cap T_2^-)$
p12	$P(T_3^- D^- \cap T_1^- \cap T_2^-)$
p13	$P(T_3^- D^- \cap T_1^- \cap T_2^+)$
p14	$P(T_3^- D^- \cap T_1^+ \cap T_2^-)$
p15	$P(T_3^- D^- \cap T_1^+ \cap T_2^+)$
p16	$P(T_4^+ D^+ \cap T_1^+ \cap T_2^+ \cap T_3^+)$
p17	$P(T_4^+ D^+ \cap T_1^+ \cap T_2^+ \cap T_3^-)$
p18	$P(T_4^+ D^+ \cap T_1^+ \cap T_2^- \cap T_3^+)$
p19	$P(T_4^+ D^+ \cap T_1^+ \cap T_2^- \cap T_3^-)$
p20	$P(T_4^+ D^+ \cap T_1^- \cap T_2^+ \cap T_3^+)$
p21	$P(T_4^+ D^+ \cap T_1^- \cap T_2^+ \cap T_3^-)$
p22	$P(T_4^+ D^+ \cap T_1^- \cap T_2^- \cap T_3^+)$
p23	$P(T_4^+ D^+ \cap T_1^- \cap T_2^- \cap T_3^-)$
p24	$P(T_4^- D^- \cap T_1^- \cap T_2^- \cap T_3^-)$
p25	$P(T_4^- D^- \cap T_1^- \cap T_2^- \cap T_3^+)$
p26	$P(T_4^- D^- \cap T_1^- \cap T_2^+ \cap T_3^-)$

p27 $P(T_4^- | D^- \cap T_1^- \cap T_2^+ \cap T_3^+)$

p28 $P(T_4^- | D^- \cap T_1^+ \cap T_2^- \cap T_3^-)$

p29 $P(T_4^- | D^- \cap T_1^+ \cap T_2^- \cap T_3^+)$

p30 $P(T_4^- | D^- \cap T_1^+ \cap T_2^+ \cap T_3^-)$

p31 $P(T_4^- | D^- \cap T_1^+ \cap T_2^+ \cap T_3^+)$

3 *p1 to p31: conditional probabilities 1 to 31

4 **P()=Probability of being (),

5 D^+ : Pig infected by *Mycoplasma hyopneumoniae*, D^- : Pig not infected by *Mycoplasma hyopneumoniae*

6 T_x^+ : Positive nested-PCR result for sampling method x, T_x^- : Negative nested-PCR result for sampling
7 method x,

8 T_1 : Nasal swabbing, T_2 : Oro-pharyngeal brushing, T_3 : Tracheo-bronchial washing, T_4 : Tracheo-
9 bronchial swabbing

10 e.g.: $P(T_1^+ | D^+)$ is the probability that the pig tested positive by nasal swabbing when the pig is
11 infected by *M. hyopneumoniae*

12

13 Table 2: Description of the assumptions made for prior definition of the parameters to be estimated in
 14 the 3 different models that were compared

parameters *	Models**		
	M1	M2	M3
p1	0-1	>0.5, 0.7	>0.5, 0.7
p2	0-1	0-1	>0.1, 0.25
p3	1	1	1
p4	0-1	0-1	>0.4, 0.8
p5	0-1	0-1	>0.2, 0.4
p6	1	1	1
p7	-	-	-
p8	0-1	0-1	>0.6, 0.8
p9	0-1	0-1	>0.1, 0.15
p10	0-1	0-1	>0.5, 0.7
p11	0-1	0-1	>0.2, 0.4
p12	1	1	1
p13 to p15	-	-	-
p16 to p24	0-1	0-1	0-1
p25 to p31	-	-	-

15 *p1,...,p31 are the conditional probabilities to be estimated; ** priors: 95 % sure >value, mode ; 0-1:
 16 uniform distribution ; 1: fixed parameter; -:not estimated

17 Table 3: Detection of *M. hyopneumoniae* in nasal swabbing, oral-pharyngeal brushing, tracheo-
 18 bronchial swabbing and tracheo-bronchial washing obtained from Specific Pathogen Free pigs
 19 (negative control group) and experimentally infected pigs (positive control group) in preliminary studies
 20 {Marois, 2007 #502} {Marois, in press #645}

Group	Sampling techniques*			
	Nasal Swab	Oro-pharyngeal Swab	Tracheo-bronchial Washing	Tracheo-bronchial Swabbing
Negative control ¹	0/15	0/5	0/10	0/5
Positive control ²	4/30 (13)	13/20 (65)	7/10 (70)	10/15 (67)

21 *: Number of positive pigs/Number of tested pigs (percentage within brackets)

22 1: By culture and PCR methods

23 2: SPF pigs intratracheally infected with 10^9 UCC of *M. hyopneumoniae*, 21 days post-inoculation,
 24 isolation of *M. hyopneumoniae* by bacteriological culture, all the pigs were not sampled with all the
 25 sampling methods which led to different total numbers

26

27 Table 4: Cross-classified test results obtained by four sampling methods for the detection of
 28 *Mycoplasma hyopneumoniae* in 60 pigs (+ indicates positive result; - indicates negative result)

Sampling method				
Nasal Swab	Oro-pharyngeal Swab	Tracheo-bronchial Washing	Tracheo-bronchial Swabbing	Number of pigs
-	-	-	-	18
-	-	-	+	7
-	-	+	-	2
-	-	+	+	8
-	+	-	-	1
-	+	-	+	1
-	+	+	-	2
-	+	+	+	13
+	-	-	-	1
+	-	-	+	0
+	-	+	-	0
+	-	+	+	0
+	+	-	-	0
+	+	-	+	0
+	+	+	-	0
+	+	+	+	7

29

30

31 Table 5: Model comparison based on the Deviance Information Criterion (DIC), the number of
32 parameters (p_D) and a Bayesian p-value (Bayes-p)

33

Model	DIC	Bayes-p	p_D
M1 (vague priors on Se, Sp of the 4 tests=1)	47.6	0.51	7.9
M2 (mildly informative priors on the prevalence, Sp of the 4 sampling methods=1)	47.4	0.51	7.7
M3 (mildly informative priors on the prevalence and parameters to estimate, Sp of the 4 sampling methods=1)	47.2	0.54	6.1

34

35

36 Table 6: Mean and 95 % Credibility Interval of posterior distributions of the sensitivity of the four
 37 sampling methods of *Mycoplasma hyopneumoniae* detection by nested-PCR, according to the 3
 38 models with different prior distributions (60 pigs sampled, specificity=1 for all models and sampling
 39 methods)

	Nasal	Oro-pharyngeal	Tracheo-bronchial	Tracheo- bronchial
Model*	Swabbing	Brushing	Washing	Swabbing
M1	0.19 (0.09-0.31)	0.51 (0.35-0.67)	0.66 (0.49-0.81)	0.72 (0.55-0.86)
M2	0.19 (0.09-0.32)	0.53 (0.38-0.68)	0.68 (0.53-0.82)	0.74 (0.59-0.86)
M3	0.25 (0.14-0.37)	0.53 (0.39-0.65)	0.62 (0.51-0.73)	0.73 (0.59-0.84)

40 *: M1: vague priors on sensitivities, specificity of the 4 tests=1, M2: mildly informative priors on the
 41 prevalence, specificity of the 4 sampling methods=1, M3: mildly informative priors on the prevalence
 42 and parameters to estimate, specificity of the 4 sampling methods=1

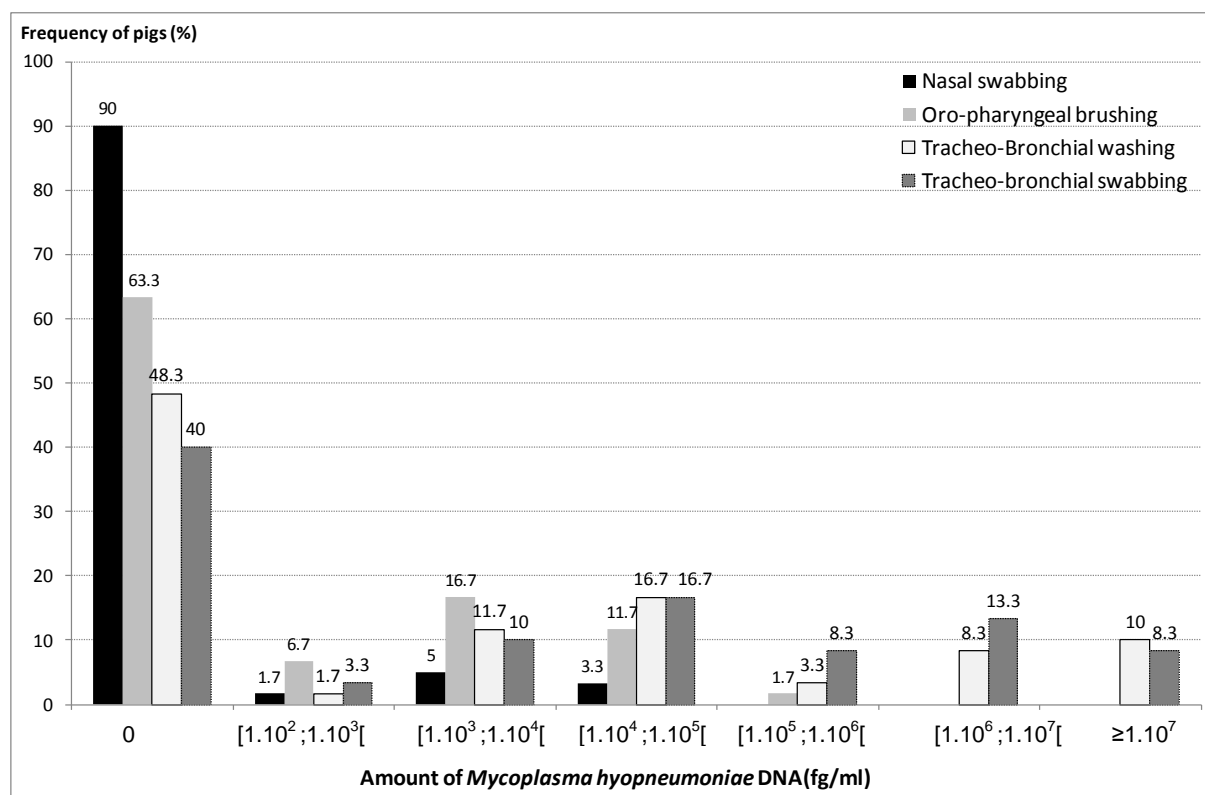
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1 Figure 1: Distribution of the amount of *Mycoplasma hyopneumoniae* DNA estimated by RT-PCR assay
 2 according to the sampling method used (60 pigs, four sampling methods)

3



4
5
6