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1 **Correlation between clinico-pathological outcome and typing of**

2 ***Haemophilus parasuis* field strains**

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22 Short Title: Pathology of *H. parasuis* field strains

23

24 **Abstract**

25 *Haemophilus parasuis* is the etiologic agent of Glässer's disease in pigs, which is
26 pathologically characterized by serofibrinous polyserositis and arthritis. *H.*
27 *parasuis* include virulent and non-virulent strains and confirmation of virulence in
28 *H. parasuis* is still dependent on experimental reproduction of the disease. Since
29 the variability in virulence is supported by serotyping and genotyping
30 (particularly, multilocus sequence typing [MLST]), we examined the relationship
31 between the classification of 8 field strains by these methods and their capacity to
32 cause disease in snatch-farrowed, colostrum-deprived piglets. The severity of
33 clinical signs and lesions produced by the different strains correlated with the
34 quantity of *H. parasuis* recovered from the lesions. However, the virulence of the
35 strains in the animal model did not show a total correlation with their serovar or
36 their classification by MLST. More studies are needed to identify a virulence
37 marker that could substitute animal experimentation in *H. parasuis*. In addition,
38 we reproduced disease in domestic pigs with a strain isolated from the nasal
39 cavity of wild boars. This result indicates the existence of virulent strains of *H.*
40 *parasuis* in wild suids, which could produce disease under appropriate
41 circumstances, and suggests a possible source of infection for domestic pigs.

42

43 **Key words:** Glässer's disease / animal model / field strains / *Haemophilus*44 *parasuis* / wild boar

45

46 1. Introduction

47 *Haemophilus parasuis* is the etiologic agent of Glässer's disease in pigs, which
48 causes high morbidity and mortality in naive swine populations and is
49 pathologically characterized by serofibrinous to fibrinopurulent polyserositis,
50 arthritis and meningitis (Rapp-Gabrielson et al., 2006). *H. parasuis* strains are
51 heterogeneous and display a diverse degree of virulence. It is also well known that
52 *H. parasuis* can be isolated from the upper respiratory tract of healthy pigs as part
53 of their normal respiratory microbiota (Harris et al., 1969; Møller and Kilian,
54 1990; Olvera et al., 2006a, 2006b). Therefore, the differentiation between virulent
55 and commensal strains would improve the diagnosis and control of disease.

56 Although several studies have identified putative virulence-associated genes, their
57 role in pathogenesis has not been demonstrated (Melnikow et al., 2005; Metcalf
58 and MacInnes, 2007; Jin et al., 2008; Chen et al., 2009; Pina et al., 2009; Sack and
59 Baltes, 2009). Thus, no definite determinant of virulence has been reported so far
60 and the confirmation of the pathogenic potential of *H. parasuis* isolates still relies
61 on the experimental reproduction of Glässer's disease.

62 Classically, *H. parasuis* strains were classified by serotyping (Kielstein and Rapp-
63 Gabrielson, 1992), but the correlation with virulence is not clear (Nielsen, 1993;
64 Amano et al., 1994; Takahashi et al., 2001; Bak and Riising, 2002). On the other
65 hand, sequencing studies revealed two clades of strains in *H. parasuis* (Olvera et
66 al., 2006a, 2006b; Angen et al., 2007) . In particular, multilocus sequence typing
67 (MLST) showed one cluster associated with systemic disease and a second cluster
68 associated with nasal isolation (Olvera et al., 2006b). This classification was

69 supported by the clinical origin of the strains, but it could not be complemented
70 with data from experimental infections.

71 Thus, the aim of this study was to analyze the relationship between the severity of
72 disease and the classification of different *H. parasuis* field strains by serotyping
73 and genotyping. Additionally, we tested the ability of a *H. parasuis* strain isolated
74 from the nose of a wild boar (*Sus scrofa*) to produce disease in domestic pigs.

75

76 **2. Materials and Methods**

77 **2.1. Bacterial strains**

78 *H. parasuis* strains used in this work and their main characteristics are
79 summarized in Table 1. Seven field strains isolated from domestic pigs and one
80 nasal isolate from wild boar were included in the study. Virulent reference strain
81 Nagasaki was also included as positive control.

82 **2.2. Animals**

83 All procedures involving animals were approved by the Ethical and Animal
84 Welfare Committee from the Universitat Autònoma de Barcelona and followed
85 EU normative (Council Directive 86/609/EEC). To avoid antibody interference,
86 snatch-farrowed and colostrum-deprived piglets were used for the infection
87 (Blanco et al., 2004). The animal model was tested in a preliminary experiment
88 with 5 animals and a field strain.

89 Piglets used in this study came from a farm with a standard health status. Briefly,
90 sows were seropositive against porcine reproductive and respiratory virus
91 (PRRSV) and swine influenza, and seronegative against Aujeszky's disease virus.

92 Piglets were obtained at the moment of the delivery, immediately dried and
93 umbilical cords were clamped, cut and disinfected with an iodine solution.

94 Piglets were housed in the biosecurity level 3 animal facilities at the Centre de
95 Recerca en Sanitat Animal (CRESA). Temperature was maintained at 28-29°C
96 from day 0 to 30 and at 24-25°C from day 30 until the end of the trial. From 8 am
97 to 8 pm, boxes were illuminated, while from 8 pm to 8 am, they were maintained
98 in darkness.

99 Piglets were fed the milk substitutive Patavie Porc (Oriane-Celtilait)
100 supplemented with antibiotics (see below) *ad libitum* during the first 2 days.
101 Afterwards, animals received Startrite 100 (SCA Ibérica S.A.) mixed with the
102 milk or dry after 10-15 days of age.

103 It was decided to administer antibiotics prophylactically to the piglets in order to
104 reduce the likelihood of appearance of bacterial disease in such a susceptible pig
105 population. Briefly, piglets received 205.000 UI of colistin (1 gr of colimicine®,
106 Laboratios SP Veterinaria, in 1.5 L of milk) during the first week and 1.7 mg of
107 enrofloxacin (Baytril solution oral 0.5%, Bayer Animal Health) per kg body
108 weight/day during the first two weeks, through the milk and by oral bolus
109 administration, respectively. After this period of prophylactic antibiotic treatment,
110 only piglets showing signs of disease (mainly diarrhea) were treated with
111 cephalosporin (Ceftiofur®, Pfizer Animal Health) or amoxicillin (Penimox®
112 Bayer Animal Health) depending on the bacteriological findings and following
113 each summary of product characteristics. In any case, antibiotic treatments were
114 stopped at least 2 weeks before experimental infection.

115 **2.3. Inoculation**

116 Bacterial suspensions for inoculation were made in PBS from overnight cultures
117 of *H. parasuis* on chocolate agar plates. Suspensions were adjusted to O.D. of 0.2
118 at 660 nm to give a bacterial concentration of approximately 10^8 CFU/ml. The
119 real number of bacteria in the suspensions was confirmed by serial dilution and
120 plating on chocolate agar (Table 1).

121 At day 40 of age, groups of 5-8 piglets were intranasally inoculated with 1.5 ml
122 (0.75 ml per nostril) of the corresponding bacterial suspension using a plastic
123 syringe. The groups were balanced according to weight.

124 **2.4. Clinical evaluation**

125 Animals were individually examined for the 6-7 days of the trial and clinical signs
126 were recorded daily. Fever, lethargy, and mortality were expected after
127 experimental infection with *H. parasuis*. Animals showing signs of suffering
128 before the end of the experiment were euthanized by an intravenous sodium
129 pentobarbital overdose (200 mg/kg, Dolethal, Vetoquinol SA). Surviving piglets
130 were euthanized at 6-7 days post-inoculation, with an intravenous overdose of the
131 mentioned product.

132 Mortality associated to *H. parasuis* infection was confirmed by post-mortem
133 examination and bacterial isolation from the lesions (see below).

134 Body weight was measured daily and average daily weight gain for the whole
135 period was calculated. After inoculation, rectal temperature was also measured
136 daily.

137 **2.5. Pathological examinations**

138 All pigs were necropsied for evaluation of gross lesions. Special attention was
139 drawn to potential features attributable to *H. parasuis* infection, such as ascites,
140 hydrothorax, and presence of fibrin in serosal surfaces, including meninges and
141 joints. Since evidence of meningeal damage is poorly visible grossly, samples of
142 brain (cerebellum and pons) of all studied pigs were taken, fixed by immersion in
143 10% buffered formalin, and routinely processed for histopathology. Severity of
144 microscopic lesions (meningitis) was scored from 0 (no lesion) to 3 (severe
145 meningitis).

146 **2.6. *H. parasuis* detection**

147 Duplicated swabs for bacterial isolation and DNA extraction were collected at
148 necropsy from nose, thoracic, pericardial and peritoneal cavities, joints and
149 meninges. Samples from pleural, pericardial, peritoneum or joint fluids were
150 taken when fluid accumulation was found. Additionally, when possible, blood and
151 cerebrospinal fluid were also processed for bacterial isolation. *H. parasuis*
152 recovery was semi-quantified by assigning a score of 3 to samples with confluent
153 growth, 2 to samples yielding isolated colonies (down to 20 colonies), 1 to
154 samples yielding 1 to 19 colonies and 0 to samples yielding no bacterial growth.

155 Samples for bacterial isolation were processed within 2 h after collection.
156 Bacterial isolation was performed by plating the swabs on chocolate agar and
157 incubating at 37°C with 5% CO₂.

158 Samples for DNA extraction and subsequent *H. parasuis*-specific PCR (Oliveira
159 et al., 2001) were stored at -20°C until used. DNA from selected swabs was

160 extracted with the Nucleospin® blood kit (Macherey-Nagel) following
161 manufacturer's instructions.

162 *H. parasuis* recovered from the different animals was processed for ERIC-PCR
163 analysis (Oliveira et al., 2003; Olvera et al., 2006a) to confirm recovery of the
164 corresponding inoculated strain.

165 ***2.7. Typing of H. parasuis by serotyping and multilocus sequence typing*** 166 ***(MLST)***

167 Serotyping determination was performed by indirect hemagglutination at the
168 Department of Sanidad Animal of the Veterinary School at the University of Leon
169 (Spain) following a previously published protocol (Del Rio et al., 2003).

170 MLST analysis of the strains included in this study was already published or
171 performed as previously described (Olvera et al., 2006b). We also deduced *in*
172 *silico* the alleles of the two *H. parasuis* genomes available in the nucleotide
173 database of NCBI (Genbank accession numbers NC_011852 and
174 NZ_ABKM00000000). Results were analyzed by BURST and unweighted pair
175 group method with arithmetic mean (UPGMA) using START2 software (Jolley et
176 al., 2001). Clusters were established by the analysis of the clinical data rather than
177 setting an arbitrary threshold.

178 ***2.8. Statistical analysis***

179 Experimental groups were compared with ANOVA followed by Tukey-Kramer
180 post hoc test for multiple comparisons of unpaired observations (average daily
181 weight gain and rectal temperature) or using Student's T test (recovery of *H.*
182 *parasuis* at necropsy). The significance level was established at 0.05, unless

183 otherwise stated. Analyses were carried out with the NCSS 2004 and PASS 2005
184 (Kavysville, Utah, USA).

185

186 **3. Results**

187 **3.1. Preliminary study: reproduction of disease**

188 Initially, we performed a preliminary trial to test the animal model (snatch-
189 farrowed and colostrum-deprived piglets) with *H. parasuis* field strains. In this
190 first trial, we used 5 piglets to intranasally inoculate a bacterial suspension of the
191 systemic isolate *H. parasuis* 264/99 (7.5×10^8 CFU/animal). Two days after
192 inoculation, 3 animals were euthanized because they showed severe clinical signs
193 (prostration, with swollen joints, and respiratory distress). At necropsy, animal 1
194 had catarrhal-purulent bronchopneumonia, animal 2 had catarrhal-purulent
195 bronchopneumonia and fibrinous polyserositis and animal 3 had fibrinous
196 pericarditis and polyarthritis. *H. parasuis* was recovered in pure culture from the
197 lesions of all 3 animals. The remaining 2 pigs survived the challenge and were
198 sacrificed at day 3 post-inoculation, although they did not show clinical signs.
199 One of these animals did not show lesions at necropsy and no *H. parasuis* was
200 recovered from the samples taken from it. From the second animal, *H. parasuis*
201 was isolated in low quantity from thorax and joints, although no macroscopic
202 lesions were observed.

203 Thus, we confirmed that we could reproduce the disease by intranasal inoculation
204 with a field strain using snatch-farrowed, colostrum-deprived piglets.

205

206 **3.2. Animal infection with *H. parasuis* field strains from domestic pigs**

207 **3.2.1. Clinical signs and pathological findings**

208 In the infections, the most prominent result was that none of the field strains was
209 able to produce the hyperacute outcome that was produced by the Nagasaki strain.
210 This outcome could be explained by the slightly higher inoculum used for this
211 strain or by its higher virulence. Table 2 shows the mortality after inoculation of
212 the different strains. Animals inoculated with strain Nagasaki died in 24h with
213 signs of septic shock. Strains PV1-12 and IT29205 produced severe disease in the
214 animals with high mortality, while strains 9904108, CA38-4 and 2725 generated
215 no mortality in the piglets. Strain 373/03A caused the death of one of the 6
216 animals infected.

217 These general observations occurred concomitantly with other clinical findings,
218 such as increased rectal temperature (Table 3) and reduced daily weight gain (Fig.
219 1) in the affected animals. Infection by strains Nagasaki, IT29205 and PV1-12,
220 which produced severe disease, produced a clear reduction in daily weight gain of
221 the piglets (Fig. 1). Following infection, elevated temperature ($\geq 41^{\circ}\text{C}$) was a
222 good predictor of the severity of disease and death of individual animals.

223 Besides, the severity of clinical signs correlated with the gross lesions observed in
224 the animals at necropsy (Table 4). Interestingly, 3 of the 5 animals infected with
225 the Nagasaki strain showed lesions characteristic of Glässer's disease (fibrinous
226 serositis), although they died within 24h after inoculation. All the animals infected
227 with IT29205 or PV1-12 also showed lesions of Glässer's disease. In the
228 surviving PV1-12-infected piglet, lesions were mild. Animals infected with

229 994108 and 2725 showed no or very mild lesions. Strain CA38-4 only produced
230 lesions (arthritis) in 3 of the 7 inoculated animals and strain 373/03A produced
231 severe lesions of fibrinous polyserositis (pleuritis, pericarditis and arthritis) in 1 of
232 the 6 inoculated animals and arthritis in an additional animal. Histopathologically,
233 2 pigs inoculated with the IT29205 strain had severe fibrino-purulent meningitis;
234 two pigs inoculated with strain PV1-12 and one pig that received the Nagasaki
235 strain showed very mild meningitis with few inflammatory cells. No microscopic
236 brain lesions were observed in the rest of the pigs inoculated with the
237 abovementioned or other strains.

238 **3.2.2. Recovery of *H. parasuis* from the inoculated animals**

239 Recovery of *H. parasuis* from the lesions of inoculated pigs with the different
240 strains is presented in Fig. 2. Animals showing severe lesions yielded higher
241 quantity of *H. parasuis* from their lesions than animal with mild lesions. Thus,
242 bacterial recovery from lesions was higher (Student's T test, $P < 0.01$) in animals
243 infected with strains Nagasaki, PV1-12 and IT29205 than with the rest of strains.
244 Taking together the data from all groups, the organ that yielded more positive
245 samples was thorax, followed closely by LCR, joints, blood and abdomen.
246 Pericardium and meninx produced the lowest number of positive samples.
247 Recovery of *H. parasuis* from the nose of diseased animals was faint, while it was
248 recovered in high quantities from the nose of animals that remained healthy
249 during the trial. This observation was sustained even with animals inoculated with
250 the same strain but producing different clinical outcomes. As an example, strain
251 373/03A produced disease in 1 piglet and *H. parasuis* recovery from the nose of

252 this animal was not possible (although it was detected by specific PCR), while it
253 was abundant from the nose of pigs inoculated with the same strain that remained
254 healthy. Similar results were obtained with strain PV1-12. An exception to this
255 observation was the lack of recovery of strain 2725, which was not detected in
256 any of the inoculated pigs at the end of the trial by bacterial culture or PCR.
257 By ERIC-PCR, it was confirmed that the *H. parasuis* recovered from the pigs
258 were effectively the same strain that we inoculated in each animal (data not
259 shown).

260 **3.2.3. Relationship between the severity of disease and the serovar of the** 261 **inoculated strain**

262 We did not see a direct correlation between the serovar and the virulence of all the
263 strains.

264 As expected, strain Nagasaki, which is the reference strain from the high virulent
265 serovar 5, produced a hypervirulent outcome in the piglets, confirming previous
266 reports with strains from serovar 5 (Kielstein and Rapp-Gabrielson, 1992;
267 Nielsen, 1993; Amano et al., 1994, 1996; De la Fuente et al., 2009). Since
268 Nagasaki was the only strain from serovar 5 included in our inoculations, this can
269 explain the higher virulence observed with this strain. We also found agreement
270 with the putative virulence of strains 264/99, IT29205 and PV1-12. These three
271 strains were originally isolated from clinical lesions, belonged respectively to
272 virulent serovars 10, 4 and 15, and were able to reproduce Glässer's disease in our
273 animal model. Conversely, strains 2725 and 9904108, which belonged also to
274 virulent serovars 10 and 4, respectively, did not produce disease. Thus, strains

275 with the same serovar showed different degree of virulence. In addition, strain
276 CA38-4, from virulent serovar 12, did not induce severe disease. Finally, strain
277 373/03A, which belonged to the non-virulent serovar 7, produced severe disease
278 in 1 piglet.

279 **3.2.4. Relationship between the severity of disease and the classification of the** 280 **strains by MLST**

281 A total of 204 sequence types (ST) were defined by MLST of 260 strains and,
282 using relaxed BURST criteria (strains sharing 5 identical loci were assigned to the
283 same group), they were assembled in 33 groups, while 102 isolates remained as
284 singletons. Searching for more distant relationships by UPGMA, 3 main clusters
285 were defined as before (supplementary figure). Strains 264/99, IT29205, CA38-4,
286 373/03A, 2725 and Nagasaki were included in cluster A, which was associated to
287 Glässer's disease production and was constituted mainly by isolates from systemic
288 lesions. Accordingly, strains 264/99, IT29205, 373/03A and Nagasaki were all
289 able to produce disease in the animal model, but the severity of disease differed
290 among strains. Moreover, strains 2725 and CA38-4, from the same virulent
291 cluster, did not produce severe disease. On the other hand, cluster B contained
292 half of the strains from Glässer's disease or pneumonia cases, but it did not show
293 a clear link with virulence. Strain PV1-12 was included in this cluster B and was
294 able to produce disease. Finally, cluster C was composed of strains isolated from
295 the nose of healthy animals. Strain 9904108 was included in this cluster C and, in
296 agreement, it did not produce disease. Thus, the correlation between the MLST
297 classification and the reproduction of disease was not complete.

298

299 **3.3. Animal infection with a *H. parasuis* strain from wild boar**

300 Noteworthy, strain WB21-4, which was isolated from the nose of a wild boar, was
301 able to produce disease in the animal model used. Disease caused by this strain
302 was found only in 1 piglet, which died from the infection and showed fibrinous
303 multifocal pleuritis and slight ascites (approximately 10 ml) at necropsy (Fig. 1,
304 Table 2, 3 and 4). This animal had also mild meningitis, while the rest of the pigs
305 showed no gross or microscopic brain lesions. *H. parasuis* WB21-4 was isolated
306 in high quantities from thoracic, pericardial and abdominal cavities, as well as
307 from brain, joints and cerebrospinal fluid from this animal (Fig. 2). The quantity
308 of inoculated strain isolated from the nose was very faint in this animal, while it
309 was extremely high (scores of 3) in the animals that remained healthy after
310 inoculation with the same strain.

311 Strain WB21-4 was classified by MLST in the nasal cluster C, but it was able to
312 produce disease in 1 animal. Serotyping result was more consistent with the
313 clinico-pathological outcome in this strain, since it was classified in the
314 moderately virulent serovar 2.

315

316 **4. Discussion**

317 We confirmed that snatch-farrowed colostrum-deprived piglets can be used for the
318 reproduction of Glasser's disease by intranasal inoculation of field strains of *H.*
319 *parasuis*. Reproduction of disease was strain-dependent and severity of clinical
320 signs and lesions correlated with the quantity of *H. parasuis* recovered from the

321 lesions of sick animals. In fact, we found a good correlation between the severity
322 of the lesions and the quantity of *H. parasuis* recovered from them, independently
323 of the organ affected. Our results indicate that the best sampling site for *H.*
324 *parasuis* diagnosis is not specific of serovar, as suggested by others (Turni and
325 Blackall, 2007), but any organ affected by the infection where lesions are
326 observed.

327 Although we observed a good agreement between the genetic classification by
328 MLST of Nagasaki, 264/99, IT29205, PV1-12 and 9904108 and the virulence of
329 these strains in the animal model, this was not the case for the other strains.
330 Besides, we found that serovar was not a good predictor of the virulence of the
331 field strains. Actually, the relationship between serovar and virulence in *H.*
332 *parasuis* is not well established, since the virulence of some serovars was
333 determined using only the reference strain of each serovar (Kielstein and Rapp-
334 Gabrielson, 1992; Rapp-Gabrielson et al., 2006). From our results and those of
335 other authors (Nielsen, 1993; Amano et al., 1994; Takahashi et al., 2001; Bak and
336 Riising, 2002), it is clear that serovar does not determine the virulence and
337 additional factors are probably responsible for virulence in *H. parasuis*. In
338 agreement, Bouchet et al. (Bouchet et al., 2008, 2009) found only a minor role of
339 LOS (one of the main antigen that determines the serovar in *H. parasuis*) in
340 invasion or release of cytokines from endothelial and epithelial cells. Although
341 several studies have tried to identify virulence factors by different methods (Hill
342 et al., 2003; Melnikow et al., 2005; Metcalf and MacInnes, 2007; Bouchet et al.,
343 2008; Jin et al., 2008; Sack and Baltes, 2009), virulence factors of *H. parasuis* are

344 still inadequately defined. Further studies are needed to identify essential
345 virulence factors that will allow a clear differentiation between virulent and non-
346 virulent strains of *H. parasuis*.

347 Besides, other aspects, such as the number of *in vitro* passages of the strains,
348 could affect the virulence status of the strains (Olvera et al., 2009) and, therefore,
349 of the inoculum used for the infections. Thus, strains with the proper genetic
350 potential for full virulence could lose part of the phenotypic characteristics and
351 appear in the animal model as poorly virulent. It is always recommended to use
352 strains with few *in vitro* passages when studying virulence-related properties. In
353 this study, no information on the number of *in vitro* passages of strains 264/99,
354 9904108, IT29205 and 2725 was available, and this could have affected the
355 results of the infection. To emphasize the importance of the inoculum, it is
356 noteworthy that reduction of the Nagasaki inoculum to 7.0×10^5 CFU per animal
357 produced no clinical signs or lesions in the piglets (data not shown).

358 Interestingly, it seemed that the strains that proceeded to invade were not
359 maintained well in the nasal cavities of the piglets. This suggests changes in the
360 bacteria from a “colonizing state” to an “invasive state”, which could be
361 modulated by the ability of each animal to control the infection. This phenomenon
362 deserves deeper study since it could have an impact, not only in the basic
363 understanding of the pathogenesis of *H. parasuis*, but also in the control of the
364 disease.

365 Finally, Glässer’s disease has never been described in wild boar, but a strain
366 isolated from the nose of wild boars was able to reproduce characteristic lesions in

367 one domestic pig. This result indicates the existence of virulent strains in wild
368 boar, which could produce disease under the appropriate conditions (such as
369 intensive farming and early weaning). The possibility of transmission of *H.*
370 *parasuis* from wild boar to domestic pigs must be, therefore, taken into account.

371

372 In conclusion, snatch-farrowed colostrum-deprived piglets can be used to
373 reproduce Glässer's disease with field strains of *H. parasuis* by intranasal
374 inoculation. The severity of disease correlated with high mortality, severe lesions
375 and recovery of high quantities of *H. parasuis* from the lesions. The virulence of
376 the strains in the animal model did not show a total correlation with their serovar
377 or their classification by MLST. Thus, further studies will be needed to find a
378 clear virulence marker, which will eliminate the need of the animal model for
379 testing the virulence of *H. parasuis* strains.

380

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387

388 **Conflict of interest statement**

389 None of the authors of this paper has a financial or personal relationship with
390 people or organisations that could inappropriately influence or bias the content of
391 this study.
392

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481 **Figures legends**

482

483 **Fig. 1.** Daily weight gain (g/day; mean±SD) after inoculation of different strains
484 of *H. parasuis*. Comparison was performed with the Tukey-Kramer Multiple-
485 Comparison Test. Values with different letters are significantly different ($P <$
486 0.05).

487

488 **Fig. 2.** Semiquantification of the recovery of *H. parasuis* from different internal
489 organs at necropsy. Swabs from the different organs were plated directly on
490 chocolate agar and the quantity of *H. parasuis* recovered from each sample was
491 recorded as 3 to 0, being 3 the highest quantity and 0 no *H. parasuis* recovered.
492 The addition of the scores of bacterial recovery from thorax, pericardium,
493 abdomen, brain, cerebrospinal fluid, blood and joints per each animal was plotted
494 in the graph. Statistical differences (Student's T test, $P < 0.01$) are indicated with
495 different letters.

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Table 1. Main characteristics of the *Haemophilus parasuis* strains used in this study and doses used to inoculate the animals.

Strain	Serovar	Organ of isolation	Country of isolation	Dose/animal
Nagasaki ^a	5	Meninges	Japan	1.0 x 10 ⁹
264/99	10	Joint	Spain	7.5 x 10 ⁸
PV1-12	15	Thorax	Spain	3.8 x 10 ⁸
9904108	4	Unknown lesion	Denmark	3.5 x 10 ⁸
IT29205	4	Unknown lesion	Italy	2.0 x 10 ⁸
CA38-4	12	Nasal	Spain	1.3 x 10 ⁸
373/03A	7	Abdomen	Spain	3.0 x 10 ⁸
2725	10	Joint	Germany	4.2 x 10 ⁸
WB21-4 ^b	2	Nasal	Spain	1.0 x 10 ⁸

^a Virulent reference strain.

^b This strain was isolated from a wild boar.

Table 2. Mortality caused by intranasal inoculation of different strains of *H. parasuis* in snatch-farrowed colostrum-deprived piglets.

Strain	Number of animals challenged	Number of clinically affected animals	Days after challenge until euthanasia ^a
Nagasaki	5	5	1, 1, 1, 1, 1
PV1-12	6	5	2, 3, 3, 3, 3
9904108	6	0	-
IT29205	6	6	3, 3, 3, 4, 4, 5
CA38-4	7	0	-
373/03A	6	1	5
2725	6	0	-
WB21-4	8	1	4

^a Animals showing no clinical signs were euthanized at the end of the experiment. These are not included in the table.

Table 2. Comparison of rectal temperatures (mean±SD) in piglets after intranasal inoculation with different strains of *H. parasuis*. Rectal temperatures were compared by the Tukey-Kramer Multiple-Comparison Test at the different days. Values with different superscript in a column are significantly different ($P < 0.05$).

Strains	Average and SD of rectal temperature at						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Nagasaki	41.44±0.27 ^a						
PV1-12	40.04±0.78 ^b	40.23±0.71 ^a	40.37±0 ^{a,b}	40.78±0 ^b	39.72±0 ^a	39.67±0 ^{a,b}	
9904108	39.60±0.26 ^{b,c}	39.63±0.29 ^{a,b}	39.45±0.13 ^a	39.44±0.25 ^c	39.69±0.39 ^a	39.54±0.17 ^a	
IT29205	39.56±0.13 ^{b,c}	40.97±0.55 ^{a,c}	40.84±1.09 ^b	41.52±0 ^a			
CA38-4	39.58±0.41 ^{b,c}	39.48±0.36 ^b	39.45±0.37 ^a	39.44±0.44 ^c	39.66±0.62 ^a	39.12±0.28 ^b	39.48±0.30 ^a
373/03A	39.39±0.41 ^{b,c}	39.41±0.36 ^b	40.04±0.97 ^{a,b}	39.70±0.52 ^{b,c}	39.49±0.41 ^a	39.36±0.19 ^{a,b}	39.38±0.16 ^a
2725	39.47±0.21 ^{b,c}	39.22±0.27 ^b	39.45±0.21 ^a	39.26±0.20 ^c	39.61±0.13 ^a	39.41±0.18 ^{a,b}	39.53±0.18 ^a
WB21-4	39.33±0.31 ^c	39.31±0.20 ^b	39.34±0.18 ^a	39.31±0.24 ^c	39.35±0.21 ^a	39.30±0.20 ^{a,b}	39,51±0,20 ^a

Table 3. Lesions found in the animals at necropsy. Results are indicated as the number of animals with the specific lesion out of the total animals in each group.

Strain	Day of death (mean \pm SD)	Animals with no lesions	Lesions					
			Fibrinous pleuritis and/or pericarditis	CPBN ^a	Hydro-pericardium	Fibrinous peritonitis	Serous-fibrinous arthritis	Others
Nagasaki	1 \pm 0.0	-	1/5	1/5	-	1/5	2/5	Cyanosis (2/5), pulmonar, intestinal or gall bladder oedema (4/5)
PV1-12	3 \pm 2.0	-	3/6	2/6	-	2/6	2/6	Pulmonar oedema (1/5)
9904108	6 ^b \pm 0.0	4/6	1/6 ^c	-	1/6 ^c	-	-	-
IT29205	3 \pm 0.9	-	3/6	-	4/6	4/6	5/6	Fibrinous meningitis (2/6)
CA38-4	7 \pm 0.0	4/7	-	-	-	-	3/7 ^c	-
373/03A	6.5 \pm 1. 2	4/6	1/6	-	1/6	-	2/6	-
2725	7 \pm 0.0	2/6	1/6 ^d	-	2/6 ^d	-	2/6 ^d	-
WB21-4	6.8 \pm 0. 7	2/8	1/8	-	5/8 ^d	-	-	-

^a Catarrhal-purulent bronchopneumonia

^b This trial was terminated at day 6 post-inoculation

^c Very mild lesions

^d Very mild lesions in two of them

Figure 1

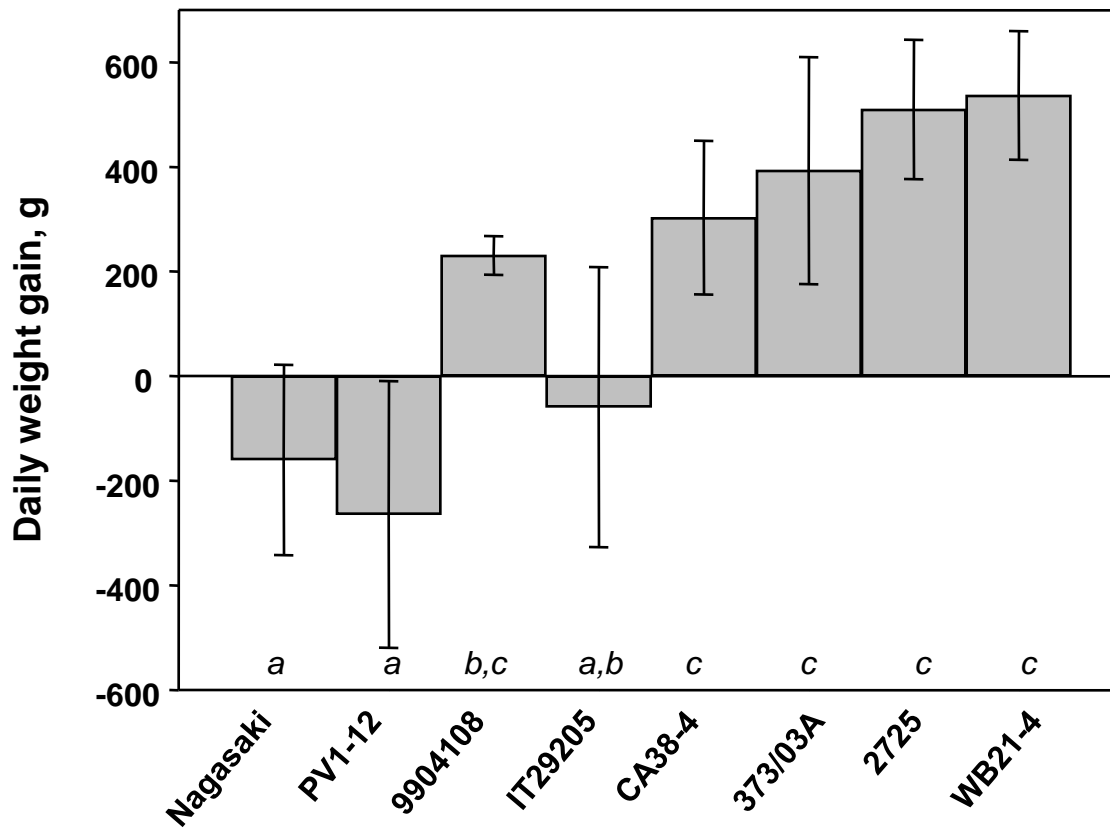


Figure 1

Figure 2

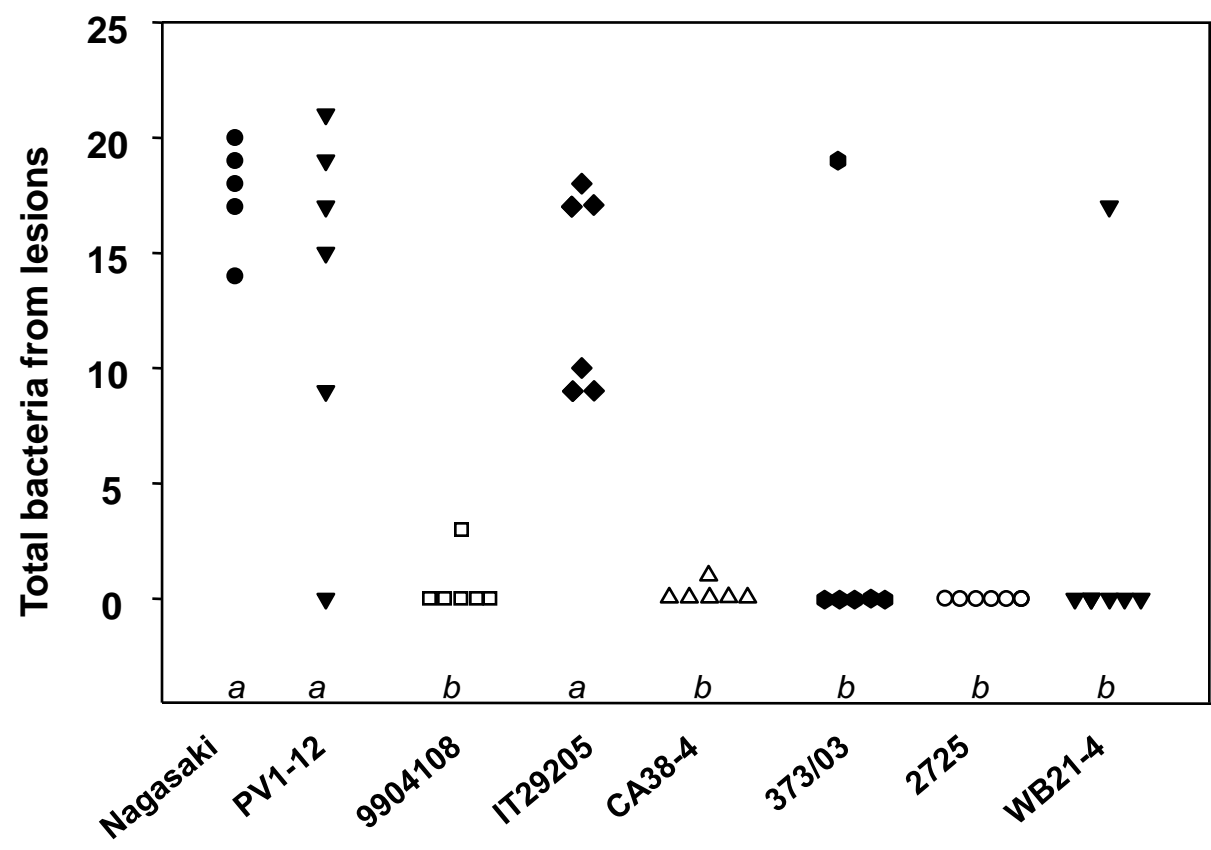
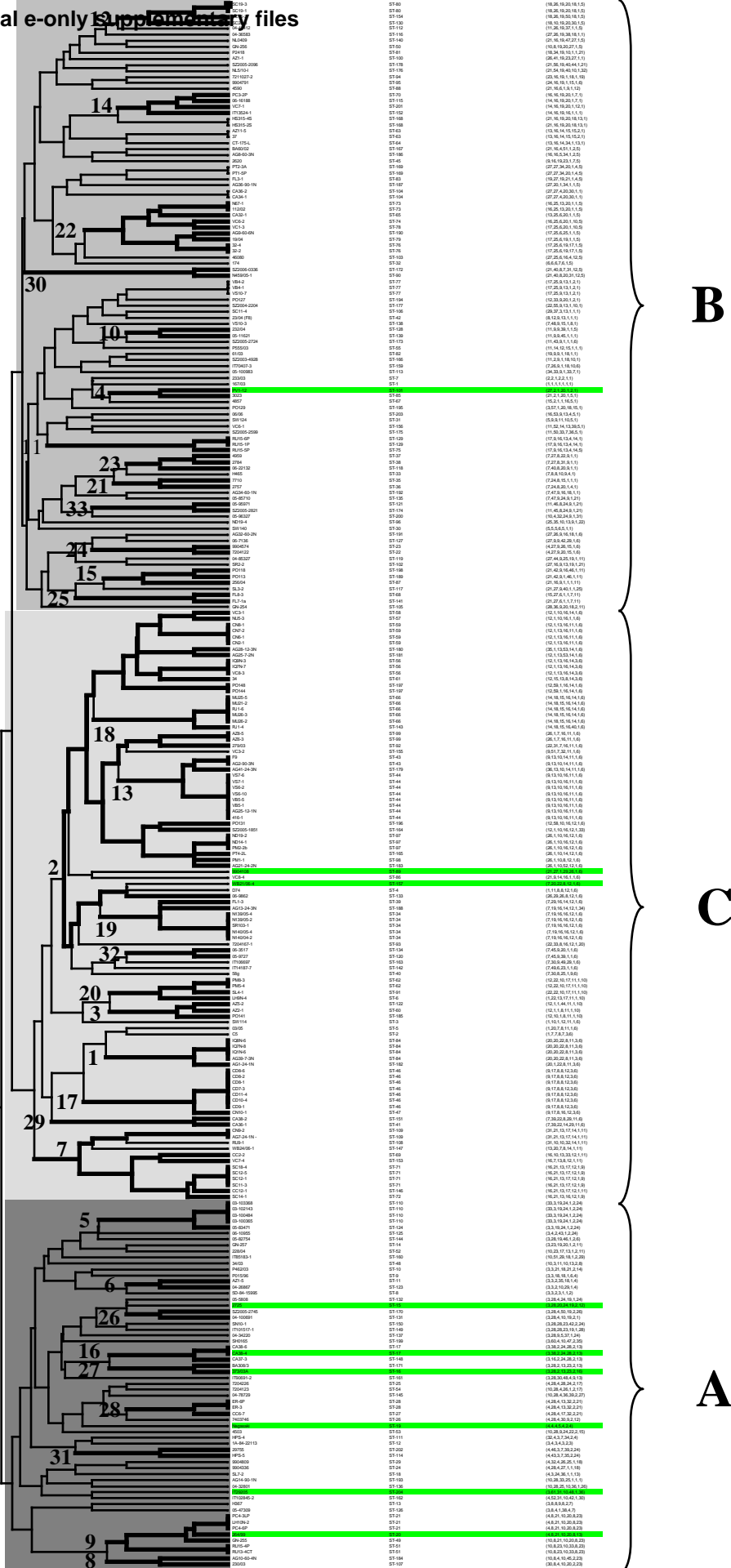


Figure 2



Supplementary figure.
UPGMA of 260
Haemophilus parasuis
 strains characterized by
 MLST. Three main
 clusters were detected
 (A,B and C). Cluster A
 contained mainly systemic
 isolates from lesions and
 cluster C contained
 mainly nasal isolates from
 healthy animals. Cluster B
 did not show any
 association with disease
 or commensalism. Groups
 detected by Burst analysis
 are indicated with a thicker
 line and the corresponding
 number. Strains used in
 this study are highlighted
 in green.

B

C

A

0.1