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Dynamics of *Haemophilus parasuis* genotypes in a farm recovered from an outbreak of Glässer's Disease

Running Title: Dynamics of *H. parasuis* strains genotypes

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24 **Abstract**

25 *Haemophilus parasuis* is a colonizer of the upper respiratory tract of pigs, although it is
26 better known as the etiological agent of Glässer's disease. Interestingly, several strains
27 can be isolated from a single farm, as determined by both genotyping and serotyping.
28 However, it is not known how an outbreak and the subsequent treatment affect the
29 population of *H. parasuis* strains. In this study, a farm was studied during an outbreak
30 of Glässer's disease and one year after antimicrobial treatment and elimination of
31 clinical signs. Bacterial isolation was attempted from nasal swabs and lesions. After
32 isolation, antimicrobial susceptibility, serotype and genotype were determined. Two
33 different genotyping techniques, enterobacterial repetitive intergenic consensus (ERIC)-
34 PCR and multilocus sequence typing (MLST) were used. The *H. parasuis* strain that
35 was isolated from lesions during the disease outbreak clustered with other virulent
36 strains by both MLST and serotyping analysis. Nasal isolates were included in the
37 corresponding nasal cluster by MLST, but they presented high variability by serotyping.
38 These nasal isolates included serotypes previously classified as virulent and non-
39 virulent. Finally, we found that during the antimicrobial treatment the diversity of
40 strains isolated in the farm was affected and just one strain, which was resistant to the
41 treatment, was detected. One year after the treatment strain diversity was back to normal
42 (3 strains).

43

44 Keywords: *Haemophilus parasuis*, Glässer's disease, field strains, genotyping

45 **Introduction**

46 *Haemophilus parasuis* is the etiological agent of Glässer's disease in pigs, which is
 47 characterized by serofibrinous to fibrinopurulent polyserositis, arthritis and meningitis
 48 (Nicolet, 1968). *H. parasuis* is also involved in other clinico-pathological outcomes,
 49 such as pneumonia and sudden death, and causes high morbidity and mortality in naïve
 50 swine populations (Miniats et al., 1986). On the other hand, *H. parasuis* colonizes de
 51 upper respiratory tract of pigs at a very young age and, therefore, it can be isolated from
 52 the nose of healthy pigs (Harris et al., 1969; Møller and Kilian, 1990; Oliveira and
 53 Pijoan, 2004).

54 *H. parasuis* strains differ in several features, including pathogenic potential (Rapp-
 55 Gabrielson et al., 2006). Classically, strains of *H. parasuis* have been classified by
 56 serotyping and, although some serotypes seem to be more pathogenic than others
 57 (Rosner et al., 1991; Rapp-Gabrielson et al., 1992; Nielsen, 1993; Amano et al., 1994,
 58 Amano et al., 1996), the correlation between serotype and virulence is not complete.
 59 More importantly, a significant percentage of isolates, between 15 and 41%, are non-
 60 typeable by serotyping (Oliveira and Pijoan, 2004). In order to circumvent this problem,
 61 fingerprinting and sequencing methods have been developed to assess the heterogeneity
 62 of *H. parasuis* isolates by genotyping (Olvera et al., 2006c). The application of
 63 enterobacterial repetitive intergenic consensus (ERIC)-PCR to *H. parasuis* is especially
 64 useful in local epidemiology (i.e. to assess the strains circulating within a farm) (Ruiz et
 65 al., 2001; Oliveira et al., 2003), but the results of this technique are hardly portable and
 66 difficult to compare among laboratories. Thus, in order to perform global epidemiology
 67 studies, other genotyping methods have been developed (Olvera et al., 2006a; Olvera et
 68 al., 2006b). Sequence typing, especially multilocus sequence typing (MLST), reported

clusters of *H. parasuis* strains associated with disease production or, on the contrary, with nasal microbiota (Olvera et al., 2006b). Using different genotyping methods, it has been shown that several strains can be isolated from a single conventional farm and that strains associated with respiratory pathology differ from systemic ones (Smart et al., 1988; Smart et al., 1993; Ruiz et al., 2001; Oliveira et al., 2003; Olvera et al., 2006a; Olvera et al., 2006b). However, it is not known how an episode of Glässer's disease and the subsequent treatment with antimicrobials affect the population of *H. parasuis* strains in a farm. Thus, the goal of this work was to study *H. parasuis* strain dynamics in a farm during an outbreak of Glässer's disease and a year later, using serotyping and two different genotyping techniques.

Material and Methods

Case farm

In October 2004, an outbreak of Glässer's disease was diagnosed in a 300-sow, farrow-to-finish farm located in Northeastern Spain. Clinical signs, including dyspnoea, diarrhoea and sudden death, were observed at about 5 weeks of age (weaning was performed at ~24 days of age). On-farm necropsies showed fibrinous polyserositis, and lungs from four affected pigs were submitted for bacterial isolation to the diagnostic service of the Department of Infectious Diseases of the Veterinary School at the Universitat Autònoma de Barcelona (UAB) in Spain. *H. parasuis* was isolated from one of these lungs (isolate 228/04), and antimicrobial sensitivity determination was performed. The veterinarian decided to start a treatment with amoxicillin (feed treatment, 400 ppm).

94 After one month of amoxicillin treatment (November 2004), a similar clinical-
 95 pathological problem (with fibrinous polyserositis) was still present in the farm, but
 96 affecting the growing units (9-11 week-old pigs). Therefore, the antimicrobial treatment
 97 was changed and a combination of phenoximetilpenicilin and tylosin was applied (feed
 98 treatment, 200 ppm and 100 ppm, respectively). At that time, two 9 week-old pigs
 99 (CD12 and CD13) were found dead and, at necropsy, sero-fibrinous fluids of thoracic
 100 and abdominal cavities were taken for bacterial isolation. Besides, eleven nasal swabs
 101 (CD1 to CD11), sampling both nostrils, were taken from 6 to 11 week-old pigs from the
 102 units with clinical disease. The established antimicrobial treatment apparently worked
 103 and no further cases compatible with Glässer's disease were reported.

104 One year after the initial outbreak (October 2005), the farm remained free of Glässer's
 105 disease based on clinical and pathological observations. At that time, ten nasal swabs
 106 from 6 to 8 week-old pigs (CN1 to CN10) were taken for *H. parasuis* isolation and the
 107 subsequent typing analysis.

108

109 ***H. parasuis* isolation and identification**

110 Collected swabs were placed in Amies medium and transported under refrigeration to
 111 the laboratory, where they were plated on chocolate agar to isolate colonies. After 2 to 3
 112 days at 37°C with 5% CO₂, all *H. parasuis*-like colonies were selected and subcultured
 113 for further analysis. A species-specific PCR (Oliveira et al., 2001) and classical
 114 biochemical tests (Møller and Kilian, 1990) to identify *H. parasuis* were performed,
 115 although final identification was accomplished by 16S rRNA gene sequencing
 116 (GenBank accession numbers DQ228977, DQ228980, DQ228981, DQ228983 to
 117 DQ228985, EF396294, EF396297 to EF396303). Bacterial suspensions in sterile
 118 phosphate buffer saline (PBS) were used to extract genomic DNA from each isolate.

119 The extraction was performed with the Instagene DNA purification Matrix 2 (Bio-Rad
120 Laboratories Inc., Spain) following manufacturer instructions. A fragment of the 16S
121 rRNA gene of about 1400 bp was amplified and sequenced as reported previously
122 (Olvera et al., 2006a). The resulting sequences were used in database searches using the
123 BLAST algorithm (Altschul et al., 1997). Both the NCBI
124 (<http://www.ncbi.nlm.nih.gov/BLAST>) and the Ribosomal Database Project
125 (<http://rdp.cme.msu.edu>) databases were searched. A threshold of 99% sequence
126 identity was used for species identification (Janda and Abbott, 2002).

128 **Antimicrobial susceptibility**

129 Antimicrobial susceptibility tests were performed using the Kirby-Bauer method
130 according to CLSI guideline M31-A2, with some modifications. Briefly, a 0.5
131 MacFarland suspension of each isolate was made from a 24 h culture on chocolate agar
132 plates. Each suspension was spread on chocolate agar plates using a swab and discs of
133 seven different antimicrobials (Neo-sensitabs™, Rosco Diagnostica, Denmark) were
134 placed on the plates, which were then incubated for 24 h at 37°C with 5% CO₂. The
135 antimicrobials tested were amoxicillin (30 µg), ceftifour (30 µg), enrofloxacin (10 µg),
136 doxycycline (80 µg), trimethoprim-sulphomethoxazole (5.2 + 240 µg) and tylosin (150
137 µg).

139 **Serotyping**

140 Serotype determination was performed by indirect hemagglutination at the Department
141 of Sanidad Animal of the Veterinary School at the University of Leon (Spain) following
142 a previously published protocol (del Rio et al., 2003). Soluble antigen was obtained

143 after boiling a bacterial suspension and subsequent centrifugation to eliminate insoluble
144 debris.

145

146 **Multilocus sequence typing (MLST)**

147 Fragments of the *rpoB*, *6pgd*, *mdh*, *infB*, *frdB*, *g3pd* and *atpD* genes of *H. parasuis* were
148 amplified as previously published (Olvera et al., 2006a). Amplicons were sequenced
149 using the same PCR primers and BigDye terminator v.3.1 kit, using an ABI 3100 DNA
150 sequencer (Applied Biosystems, USA). Sequences were deposited at the GenBank
151 database (accession numbers DQ781634 to DQ781637, DQ781660, DQ781661,
152 DQ781663, DQ781666, DQ781673, DQ781503 to DQ781506, DQ781529, DQ781530,
153 DQ781532, DQ781535, DQ781542, DQ782289 to DQ782292, DQ782315, DQ782316,
154 DQ782318, DQ782321, DQ782328, DQ782158, DQ782159, DQ782184, DQ782185,
155 DQ782187, DQ782190, DQ782197, DQ782027 to DQ782030, DQ782053, DQ782054,
156 DQ782056, DQ782059, DQ782066, DQ782896 to DQ782899, DQ78292, DQ78293,
157 DQ782925, DQ782928, DQ782935, DQ782765 to DQ782768, DQ782791, DQ782792,
158 DQ782794, DQ782797, DQ782804, EF396288 to EF396293, EF396308 to EF396322).
159 Sequence editing and allele assignment was performed with Fingerprinting II v3.0
160 software (BioRad, USA). MLST analysis was carried out using START2 (Jolley et al.,
161 2001) by the neighbour-joining algorithm (NJ) based on allelic profiles.

162

163 **Enterobacterial repetitive intergenic consensus (ERIC)-PCR**

164 Purified DNA was quantified by spectrometry and 100 ng were used as template in
165 ERIC-PCR. The technique followed a previously published protocol (Oliveira et al.,
166 2003), including an extra final extension step of 20 min (Olvera et al., 2006a). Images
167 of the gels were captured with a ChemiGenius transilluminator (SynGene Ltd. Frederick,

MD, USA.) and stored as TIFF files for further analysis. Bands from 4,000 to 100 bp were used in the analysis. ERIC-PCR fingerprints analysis, sequence editing and analysis were performed using the Fingerprinting II v3.0 software. Finally, ERIC-PCR band patterns were normalized and a Pearson correlation similarity matrix calculated. Cluster analysis of ERIC-PCR fingerprints were performed by UPGMA (Ooyen, 2001).

Results and discussion

Several studies have examined the variability of *H. parasuis* strains circulating in a farm by genotyping and serotyping, but none of them has followed up the strain population over time. Moreover, there are several studies on the antimicrobial susceptibility of *H. parasuis*, but, as far as we know, this is the only work that studies the effect of antimicrobial treatment on *H. parasuis* strain variability within the same farm.

When disease outbreak was observed (October 2004), *H. parasuis* was isolated from one of the 4 lungs from sacrificed animals with fibrinous polyserositis (isolate 228/04). Antimicrobial susceptibility tests showed that isolate 228/04 was sensitive to all the antimicrobials tested (Table 1). Amoxicillin treatment was implemented but, after one month (November 2004), the clinical problem moved to the growing units. Two animals (CD12 and CD13) were found dead and after necropsia, *H. parasuis* DNA was detected by PCR in abdominal and thoracic fluids. However, *H. parasuis* could not be isolated from the lesions of these pigs. These results can be explained by the fastidious growth requirements of this microorganism and its difficult isolation from already dead animals. At that time, a total of 25 *H. parasuis* isolates were obtained from eleven nasal swabs (isolates CD1 to CD11). Genotyping of these nasal isolates showed that they had the same ERIC-PCR profile (Fig. 1), no differences in 16S rRNA gene sequence (data

193 not shown) and the same MLST sequence type (ST) (Fig. 1), indicating that they were a
 194 single strain. The antimicrobial susceptibility tests of several *H. parasuis* CD isolates
 195 demonstrated that those isolates were highly resistant to amoxicillin and sensitive to the
 196 rest of antimicrobials tested (Table 1). Antimicrobial treatment had a clear effect on the
 197 population of *H. parasuis* in this farm. Clearly, the use of amoxicillin to control the
 198 outbreak selected one clone (*H. parasuis* CD) that was resistant to this antimicrobial
 199 and induced a homogenization of the strain population. Apparently, strain 228/04 was
 200 eliminated by the treatment, since it was not isolated anymore.

201 Besides antimicrobial susceptibility, *H. parasuis* CD isolates and strain 228/04 showed
 202 clear genotypic differences (Fig. 1), indicating that the *H. parasuis* CD strain and
 203 228/04 were indeed different strains. These results may indicate that two strains, 228/04
 204 and *H. parasuis* CD, were initially associated with clinical problems and the
 205 antimicrobial treatment eliminated just one or, alternatively, that the elimination of the
 206 first one (228/04) was an opportunity for the second one (CD) to proliferate and cause a
 207 second round of disease. On the other hand, it can not be ruled out that the first one was
 208 never the cause of the disease. Actually, 228/04 was isolated from lung and a clear link
 209 with the observed systemic disease is not evident, since a lung isolate may not
 210 necessarily be the cause of a septicaemia. This is a major problem with all *H. parasuis*
 211 lung isolates from Glässer's disease cases, together with the difficulties to discard them
 212 as postmortem invasion from the upper respiratory tract. The global comparison of
 213 strains by MLST (Fig 2) supported that 228/04 was indeed the cause of the disease since
 214 it was included in a cluster associated with disease production and formed mainly by
 215 systemic isolates (Olvera et al., 2006b). Moreover, this strain belonged to serotype 5,
 216 which has been repeatedly demonstrated highly virulent in experimental infections
 217 (Kielstein and Rapp-Gabrielson, 1992; Nielsen, 1993; Amano et al., 1994). On the other

218 hand, *H. parasuis* CD isolates were included in a MLST cluster with strains of nasal
 219 origin (Fig. 2), indicating that they were probably a non-virulent strain not implicated in
 220 the clinical outbreak. Curiously, when the serotype was determined for 3 of those
 221 isolates, they belonged to serotypes 10, 14 and 15; although they had the same genotype
 222 (Fig 1). Taking into account the rate of evolution of ERIC-PCR and MLST patterns this
 223 could mean that serotype in *H. parasuis* is an unstable phenotypic characteristic that can
 224 change relatively fast, at least faster than genomic rearrangements or mutations.
 225 Alternatively, *H. parasuis* serotyping needs further optimization to avoid this
 226 variability, which could be due to a certain degree of crossreactivity between serotypes.
 227 Although no other *H. parasuis* strain was isolated in the affected growing units during
 228 the clinical problem, association of *H. parasuis* CD strain with disease onset is,
 229 obviously, difficult to establish because it was isolated from nasal swabs. Unfortunately,
 230 no *H. parasuis* could be isolated from the lesions of pigs CD12 and CD13, although the
 231 bacterium was detected by the *H. parasuis*-specific PCR. Unexpectedly, ascites from
 232 pig CD12 yielded a *Moraxella spp* isolate (CD12CA4), as identified by 16S rRNA
 233 gene sequencing (best Blastn hit with *Moraxella cuniculi* [Accession number
 234 AF005188] with 97% of sequence identity). This result indicates the possibility that the
 235 clinical signs observed in the growing units were due to this opportunistic pathogen.
 236 Nevertheless, the association of *Moraxella spp* with disease onset in pigs is difficult to
 237 establish. Some authors have reported the isolation of *Moraxella*-like microorganisms
 238 from polyserositis lesions of pigs, but the pathogenicity of these isolates could not be
 239 demonstrated in experimental infections (Larsen et al., 1973; Nielsen and Danielsen,
 240 1975).

241 Finally, the treatment was changed to phenoximethylpenicillin and tylosin and the
 242 problem was controlled (all the isolates of this study were sensitive, at least, to tylosin)
 243 and the *H. parasuis* CD strain was eliminated.

244 After one year, in which the farm remained free of Glässer's disease, ten 6 to 8-week-
 245 old pigs were sampled and a total of 23 isolates of *H. parasuis* were obtained (isolates
 246 CN followed by numbers). Three different strains were identified among these CN
 247 isolates (represented by CN9-2, CN10-1 and the group of CN8-1; Fig 1) by both
 248 genotyping methods (ERIC-PCR and MLST). Thus, the variability of strains was back
 249 to normal (usually, 3-5 strains/farm) (Smart et al., 1988; Smart et al., 1989; Smart et al.,
 250 1993; Rafiee et al., 2000). When the MLST analysis was done with 205 strains from
 251 different origins, all CN isolates were included in the cluster of strains associated with
 252 nasal isolation (Fig. 2) (Olvera et al., 2006b). Serotype was determined for a
 253 representative of each genotype and serotypes 9 and 15 were reported (Fig. 1). These
 254 isolates showed different susceptibilities to amoxicillin (Table 1), but all of them were
 255 sensitive to the rest of antimicrobials tested.

256 CN10-1 was resistant to amoxicillin (Table 1) and showed the closest genotype to the
 257 CD isolates, with just one different allele (*infB* 16) in the MLST allelic profile. Allele
 258 *infB* 16 was also present in the isolates from the group of CN8-1. The CN10-1 *infB*
 259 allele (*infB* 16) differed in two nucleotides with the corresponding allele in the CD
 260 strains (*infB* 8) and was identical to the CN8-1 *infB* allele. The detection of homologous
 261 recombination in *H. parasuis* in a previous work (Olvera et al., 2006b) gives more
 262 support to the occurrence of one homologous recombination event between the CD and
 263 the CN8-1 isolates, instead of two independent point mutation events. Indeed, it is very
 264 likely that CN10-1 is a descendent of the *H. parasuis* CD strain that has exchanged the
 265 *infB* 16 allele with the CN8-1 isolates. The origin of the remaining CN strains

266 (represented by isolates CN2-1 and CN9-2) is not fully clear given that they were very
267 different from those detected during the clinical outbreak. It is possible that those CN
268 strains were in the farm prior to the outbreak. Thus, the use of antimicrobial treatment
269 induced the elimination of them in the units where the treatment was applied, but they
270 were reintroduced by the sows. Therefore, they might represent the situation of the farm
271 before the clinical outbreak. However, it cannot be ruled out that those CN strains were
272 introduced by replacement breeding stock.

273 The genotyping methods used in this study, ERIC-PCR and MLST, were useful in the
274 assessment of the number of clones present in a farm, and yielded similar results. For
275 the identification of the different clones in a farm, ERIC-PCR is faster and less
276 expensive than MLST. However, MLST produces additional information, since it also
277 allows the comparison of the isolates in a particular farm with other epidemiologically-
278 unrelated isolates. The comparison of the isolates obtained in the case farm with strains
279 from different clinical and geographical origins (Olvera et al., 2006b) was helpful to
280 elucidate the putative virulence of the strains.

281 In summary, this study indicates that the antimicrobial treatment allowed the emergence
282 of a resistant strain, which increased in frequency and resulted in the only *H. parasuis*
283 strain isolated, but the strain diversity could return to normality in less than a year after
284 the episode of Glässer's disease.

285

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292

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371

372 **Figure legends**

373 **Figure 1.** Genotyping and serotyping of *H. parasuis* isolates. ERIC-PCR fingerprints
 374 were analyzed by UPGMA and MLST allelic profiles by neighbour-joining. MLST
 375 allelic profiles correspond to the alleles of *rpoB*, *6pgd*, *mdh*, *infB*, *frdB*, *g3pd* and *atpD*.
 376 Strain 228/04 was isolated from lung lesions during a Glässer's disease outbreak, CD
 377 isolates were obtained from nasal swabs after 1 month of amoxicillin treatment and CN
 378 isolates were obtained from nasal swabs 1 year later. Isolates corresponding to a single
 379 strain are included in the same box. *ND, not determined.

380

381 **Figure 2.** Neighbour-joining dendrogram constructed using the allelic profiles of loci
 382 *rpoB*, *6pgd*, *mdh*, *infB*, *frdB*, *g3pd* and *atpD* of 205 *H. parasuis* isolates. Isolates from
 383 the case farm are indicated in the dendrogram. Additionally, non-virulent reference
 384 strains D74, SW114, C5 and virulent reference strains Nagasaki, H367, 84-22113, 84-
 385 15995 are also indicated.

386

Table 1. Antibiotic susceptibility of the different isolates studied.

Isolate	Bacteria	Antimicrobials ^{1,2}						Isolation date
		AMOXY	TR+SU	ENROF	DOXYC	CFTIO	TYLOS	
228/04	<i>H. parasuis</i>	S (52)	S (39)	S (41)	S (39)	S (57)	S (33)	10/2004
CD7-3	<i>H. parasuis</i>	R (0)	S (34)	S (34)	S (32)	S (38)	S (28)	11/2004
CD9-1	<i>H. parasuis</i>	R (0)	S (28)	S (34)	S (32)	S (38)	S (34)	11/2004
CD10-4	<i>H. parasuis</i>	R (0)	S (30)	S (36)	S (34)	S (42)	S (30)	11/2004
CD11-4	<i>H. parasuis</i>	R (0)	S (34)	S (32)	S (30)	S (38)	S (26)	11/2004
CD8-1	<i>H. parasuis</i>	R (0)	S (28)	S (36)	S (34)	S (42)	S (32)	11/2004
CN2-1	<i>H. parasuis</i>	R (12)	S (32)	S (34)	S (32)	S (38)	S (26)	10/2005
CN6-1	<i>H. parasuis</i>	I (18)	S (34)	S (34)	S (32)	S (38)	S (26)	10/2005
CN7-2	<i>H. parasuis</i>	I (17)	S (34)	S (36)	S (30)	S (42)	S (28)	10/2005
CN8-1	<i>H. parasuis</i>	I (17)	S (32)	S (36)	S (32)	S (40)	S (28)	10/2005
CN9-2	<i>H. parasuis</i>	S (34)	S (30)	S (42)	S (34)	S (36)	S (26)	10/2005
CN10-1	<i>H. parasuis</i>	R (0)	S (28)	S (34)	S (32)	S (38)	S (26)	10/2005
CD12CA4	<i>Moraxella</i> spp	R (13)	R (15)	I (18)	I (24)	S (24)	S (25)	11/2004

¹ **AMOXY**: amoxicillin (30 µg); **DOXYC**: doxycycline (80 µg); **ENROF**: enrofloxacin (10 µg);

TR+SU: trimethoprim-sulphomethoxazole (5.2 + 240 µg); **CFTIO**: ceftifour (30 µg); **TYLOS**: tylosin (150 µg).

² Inhibition diameter in mm is indicated in parenthesis.