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1 **Reappearance of *Salmonella* serovar Choleraesuis var. Kunzendorf in Danish pig herds**

2

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19

20 **Abstract**

21 *Salmonella enterica* serovar Choleraesuis is a porcine adapted serovar which may cause serious
22 outbreaks in pigs. Here we describe outbreaks of salmonellosis due to *S. Choleraesuis* in four
23 Danish pig farms in 2012 – 2013 by clinic, serology, and microbiology and compare the isolates to
24 those of a previous outbreak in 1999 – 2000. The infection was in some herds associated with high
25 mortality and a moderate to high sero-prevalence was found. In 2012 – 2013 the disease contributed
26 to increased mortality but occurred concomitant with other disease problems in the herds, which
27 likely delayed the diagnosis by up to several months. Nine isolates from the four farms in 2012 –
28 2013 and 14 isolates obtained from the outbreak in Denmark in 1999 – 2000 were subjected to
29 typing using pulsed-field gel electrophoresis (PFGE). Seven isolates were selected for whole
30 genome sequencing (WGS). The PFGE results of 23 isolates displayed five different profiles. The
31 isolates from 2012 – 2013 revealed two distinct profiles, both different from the isolates recovered
32 in 1999 – 2000. Two of the 2012 – 2013 farms shared PFGE profiles and had also transported pigs
33 between them. The profile found in the two other 2012 – 2013 farms was indistinguishable but no
34 epidemiological connection between these farms was found. Analysis of the number of single
35 nucleotide polymorphisms (SNPs) from the WGS data indicated that the isolates from the farms in
36 2012 – 2013 were more closely related to each other than to isolates from the outbreak in 1999. It
37 was therefore concluded that the infection was a new introduction and not a persistent infection
38 since the outbreak in 1999. It may further be suggested that there were two or three independent
39 rather than a single introduction. The re-introduction of *S. Choleraesuis* in Denmark emphasizes the
40 importance of strict hygiene measures in the herds. Further investigations using WGS are now in
41 progress on a larger collection of isolates to study clonality at European level and trace the origin of
42 the infections.

43 Introduction

44 Pork is one of the most important sources of human foodborne salmonellosis in the EU (EFSA,
45 2013) and the USA (Gould *et al.*, 2013). Pigs can be colonized with a variety of *Salmonella*
46 serotypes (EFSA 2008, 2009) but mostly, pigs are asymptomatic carriers. In Denmark, the most
47 common *Salmonella* serovars in pigs are *Salmonella enterica* serovar Typhimurium (*S.*
48 Typhimurium) (including monophasic *S.* 4,[5],12:i:-), *S.* Derby and *S.* Infantis (Argüello *et al.*,
49 2013, 2014). These serovars may also cause clinical salmonellosis in pigs, but the extent of clinical
50 salmonellosis in pigs in Denmark is uncertain.

51 *S.* Choleraesuis is a serovar, which is host-adapted to pigs, and may cause serious outbreaks of
52 salmonellosis and paratyphoid (Griffith *et al.* 2006). The majority of the *S.* Choleraesuis outbreaks
53 in pigs are caused by var. Kunzendorf (Fedorka-Cray *et al.*, 2000). In the USA, *S.* Choleraesuis was
54 by far the most frequently found serovar in pigs until the mid-1990'ies. In 1986, 71% of the isolates
55 from pigs were *S.* Choleraesuis, but thereafter the prevalence of this serovar declined while other
56 serovars increased, and from 1995 and onwards, *S.* Typhimurium and *S.* Derby have been most
57 prevalent (Foley *et al.*, 2007). Yet, in 2005 *S.* Choleraesuis still constituted 9% of all clinical
58 *Salmonella* isolates from pigs in the USA (Foley *et al.*, 2007). In Europe, *S.* Choleraesuis is a
59 relatively rare serovar, both in slaughter pigs and in breeding herds but it has been reported with
60 low frequency in a number of countries (EFSA, 2008, 2009). Out of 42,417 isolates from pigs and
61 pork in 2011, 695 were *S.* Choleraesuis (EFSA, 2013), but its significance as source of clinical
62 salmonellosis – human or in pigs – is not known.

63 In the USA, the disease is typically a porcine post weaning disease with septicaemia,
64 enterocolitis and pneumonia and it has been reported to occur most often in farms where pigs of
65 different ages and litters are mixed (Anderson *et al.*, 2000). *S.* Choleraesuis seems more often to be

66 isolated from non-gastrointestinal organs than other serovars, most notably from the lungs (Gray *et*
67 *al.*, 1996).

68 In humans, *S. Choleraesuis* tends to be more invasive and cause less gastrointestinal
69 manifestations than most other serotypes and thus, it is a serious infection with a significant
70 mortality (Cohen *et al.* 1987). Yet, this organism is not a common human pathogen in EU (EFSA,
71 2013) or in the USA in spite of its relatively high prevalence in American pigs (CDC, 2008). In
72 Denmark, the latest case of human infection with *S. Choleraesuis* was a var. Decatur case in June
73 2012 and before that a var. Kunzendorf case in December 2011, both travel related (Dr. Eva Møller
74 Nielsen, Statens Serum Institut, Copenhagen, personal communication). However, in Asian
75 countries, such as Thailand and Taiwan, this serovar continues to be important for human illness
76 (Chiu *et al.*, 2004; Hendriksen, 2010), although the incidences seem to be declining (Su *et al.*,
77 2014).

78 In Denmark, *S. Choleraesuis* was last found in pigs at an outbreak in 1999 (Baggesen *et al.*,
79 2000), but in 2012 and 2013 it reappeared with outbreaks of severe salmonellosis in four farms. It
80 has neither in relation to the outbreak in 1999 (Baggesen *et al.*, 2000) nor to the outbreaks in 2012
81 and 2013 been possible to identify the primary introduction of infection to the Danish pig herds.
82 This may have been due to limitations in the epidemiological information available but also by an
83 insufficient resolution of isolates by the epidemiological typing methods applied.

84 In the present study, we describe the reappearance of *S. Choleraesuis* in Danish pig farms during
85 2012 and 2013. We investigated the clonality of those isolates by the application of pulsed-field gel
86 electrophoresis (PFGE), antimicrobial susceptibility testing (MIC), and whole genome sequencing
87 (WGS), and compared to isolates from the previous Danish outbreak in 1999.

88

89 1. Material and methods

90 1.1. Farm data *Salmonella* isolates for epidemiological investigations

91 Farm data was retrieved from observations made by the Danish Pig Research Center and
92 registrations via the *Salmonella* control programme
93 (<https://www.retsinformation.dk/Forms/R0710.aspx?id=141725>). Data from the serological meat
94 juice surveillance for *Salmonella* was extracted from the Danish Zoonosis Register. The serological
95 test includes LPS antigens from *Salmonella* serovars *S. Typhimurium* and *S. Choleraesuis* and
96 covers the O factors O1, O4, O5, O6, O7 and O12 (Nielsen *et al.* 1995). The herds were assigned to
97 one of three infection levels on the basis of serological examination of meat juice samples collected
98 at the slaughterhouse and action was taken for herds reaching levels two or three (Alban *et al.*
99 2012). Serological results for the four farms were extracted for the period 2010 – 2014 in order to
100 analyse the time before, during, and after the diagnosis was made in the farms. Other farm data was
101 retrieved from the Central Herd Register (<https://chr.fvst.dk>). Twenty-three *S. Choleraesuis* isolates
102 from an outbreak on four pig farms in 2012 – 2013 (n = 9) and an outbreak in 1999 – 2000 (n = 14)
103 were included in the study and subjected to PFGE analysis. On the basis of the PFGE results, seven
104 isolates were further analysed using WGS and MIC determination, including three from the
105 outbreak in 1999 – 2000 and one from each of the outbreaks on four different farms in 2012 - 2013.

106

107 1.2. Serotyping and biotyping

108 Serotyping was performed by slide agglutination with polyclonal antisera (Statens Serum
109 Institut, Copenhagen, Denmark) according to the White – Kauffmann – Le Minor scheme (Grimont
110 and Weill, 2007) and distinction between *S. Paratyphi C*, *S. Typhisuis* and the biovars of *S.*

111 Choleraesuis, var. Kunzendorf and var. Decatur, was performed by biochemical tests (Grimont and
112 Weill, 2007).

113

114 1.3. Pulsed-field gel electrophoresis

115 PFGE was carried out according to the PulseNet protocol as previously described (Ribot *et al.*,
116 2006) using *Xba*I (Fermentas, Lifesciences) as restriction enzyme and electrophoresis carried out in
117 a Chef-DR[®]-III (Bio-Rad[®]). Banding patterns were analysed in BioNumerics[®] version 7.1 (Applied
118 Maths, Sint-Martens-Latem, Belgium) with a position tolerance of 1.5% and optimization of 1.5%.
119 Results were compared using the Dice coefficient for similarity and unweighted pair group method
120 with arithmetic averages (UPMGA) for clustering.

121

122 1.4. Antimicrobial resistance profile

123 Antimicrobial susceptibility testing was performed by Minimum Inhibitory Concentration
124 (MIC) determination using a broth microdilution method (SensiTitre system, Trek Diagnostic
125 Systems Ltd., UK) according to recommendations by the Clinical Laboratory Standards Institute
126 (CLSI 2012). Susceptibility was tested against amoxicillin-clavulanic acid, ampicillin, apramycin,
127 cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, nalidixic
128 acid, neomycin, spectinomycin, streptomycin, sulphonamides, tetracycline and trimethoprim. MIC
129 values were interpreted using EUCAST epidemiological cut-off values (www.eucast.org) with
130 exception of apramycin for which the value >16 µg/ml was used (DANMAP, 2012, 2013).

131

132 1.5. Whole genome sequencing, multilocus sequence typing, antimicrobial resistance
133 genes, plasmid replicons, and plasmid multilocus sequence typing

134 Chromosomal DNA of the subset of six *S. Choleraesuis* isolates was used to create genomic
135 libraries using the Nextera XT DNA sample preparation kit (Illumina Inc., San Diego, CA, cat. no.
136 FC-131-1024) followed by multiplexed, paired-end sequencing using a MiSeq platform (Illumina).
137 The six selected isolates are marked with an asterisk in Figure 1.

138 Raw sequence data have been submitted to the European Nucleotide Archive
139 (<http://www.ebi.ac.uk/ena>) under accession no. PRJEB5487. The fastq files are accessible from the
140 following link: <http://www.ebi.ac.uk/ena/data/view/PRJEB5487>. The raw reads were assembled
141 using the pipeline available from the Center for Genomic Epidemiology (CGE)
142 (www.genomicepidemiology.org) which is based on Velvet algorithms for *de novo* short reads
143 assembly (Zerbino and Birney, 2008).

144 The *de novo* assembled sequences were analyzed using similar pipelines available on the CGE
145 website. The web-servers, MLST version 1.7, ResFinder version 2.1 and PlasmidFinder version 1.1,
146 available at the Center for Genomic Epidemiology website (www.genomicepidemiology.org)
147 (Zankari *et al.*, 2012; Larsen *et al.* 2012; Carattoli *et al.*, 2014) were used to identify the multilocus
148 sequence type (ST) for *Salmonella enterica*, the plasmid replicons, and acquired antimicrobial
149 resistance genes with a selected threshold equal to 85 %. The identity and results of the ResFinder
150 were compared with phenotypic antimicrobial susceptibility testing results. Ribosomal MLST
151 (rMLST) types were obtained by querying the rMLST database available at
152 <http://pubmlst.org/rmlst/> (Jolley *et al.*, 2012).

153

154 1.6. Single Nucleotide Polymorphisms

155 Single nucleotide polymorphisms (SNPs) were determined using the pipeline available on the
156 Center for Genomic Epidemiology (www.genomicepidemiology.org) (Leekitcharoenphon *et al.*,
157 2012). This pipeline contains various freely available SNP analysis software. Briefly, the paired-end
158 reads from seven *S. Choleraesuis* isolates were aligned against the reference genome, *S.*
159 *Choleraesuis* strain AE017220. (National Center for Biotechnology Information, accession
160 AE017220, length of 4755700 bp), using Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009).
161 SAMtools 'mpileup' command (Li *et al.*, 2009) and BEDTools (Quinlan and Hall, 2010) were used
162 to determine and filter SNPs. The qualified SNPs were selected once they met the following
163 criteria: (1) a minimum coverage (number of reads mapped to reference positions) of 10; (2) a
164 minimum distance of 15 bps between each SNP; (3) a minimum quality score for each SNP at 30;
165 and (4) all indels were excluded.

166 The qualified SNPs from each genome were concatenated to a single alignment corresponding
167 to position of the reference genome using an in-house Perl script. In case SNPs were absent in the
168 reference genome, they were interpreted as not being a variation and the relative base from the
169 reference genome was expected (Leekitcharoenphon *et al.*, 2012). The concatenated sequences were
170 subjected to multiple alignments using MUSCLE from MEGA5 (Tamura *et al.*, 2011). The final
171 phylogenetic SNP tree was computed by MEGA5 using the maximum likelihood method (Tamura
172 and Nei, 1993) of 1,000 bootstrap replicates (Felsenstein, 1985).

173

174 **2. Results**

175 *2.1. Outbreak description*

176 The outbreaks in 2012 and 2013 occurred on four different farms, arbitrarily designated farm A,
177 B, C, and D. In addition, isolates for the investigations from an outbreak in 1999 on a farm,

178 designated farm E, were included in the typing studies. The location of the five farms is shown in
179 Figure 1 together with some information about each farm.

180 Farm A experienced a dramatic increase in *Salmonella* sero-prevalence in meat juice samples in
181 August 2012 and became classified as a level 2 herd in September and level 3 in October (Fig 2)
182 Concomitantly, there was an outbreak of oedema disease, and the mortality increased to appr. 20 %
183 among pigs up to 30 kg. Antibiotic treatment was initiated at this point, but unfortunately, no
184 samples were taken for *Salmonella* culture. The farm had previously in January 2011 been
185 diagnosed with *S. Infantis*. It was not until November 2012 that carcasses were submitted to a
186 laboratory and *S. Choleraesuis* was identified from lung tissue. The pigs were subsequently culled
187 and the premises cleaned and disinfected, and in February 2013 new SPF animals were installed.
188 Pen floor samples collected in January 2014 to follow up on *Salmonella* status did not show
189 presence of *S. Choleraesuis*, but *S. Infantis* was still present. Serological data showed that the herd
190 continued to have a low proportion of sero-positive samples (Figure 2) – possibly due to *S. Infantis*.

191 Farm B purchased pigs from farm A during the summer 2012, and when farm A was diagnosed
192 with *S. Choleraesuis*, farm B was also investigated due to the registered transfer of pig. At that time,
193 the *Salmonella* seroprevalence in the herd had changed from zero to low and the farm had
194 experienced increased mortality, and upon laboratory investigation of dead pigs, the farm was
195 diagnosed with *S. Choleraesuis* as well as *S. Derby* in January 2013. The herd was then culled;
196 some of the oldest buildings were destroyed, and the remaining were cleaned and disinfected, where
197 after the farm started to operate again. Follow-up pen floor samples collected in September 2013
198 did not show presence of *S. Choleraesuis* but both *S. Derby* and *S. Infantis* were found. Serological
199 data showed that a moderate proportion of sero-positive samples was still present (Figure 2).

200 Farm C was a family operated farm with no foreign assistance. There was a general very high
201 mortality around 20 – 30 % among the 7 – 50 kg pigs. For at least a couple of years prior to the
202 diagnosis, the herd had a high sero-prevalence for *Salmonella* in meat juice samples. Forty-one pigs
203 were in the spring 2013 sold to another herd, which subsequently experienced very high meat juice
204 sero-prevalence and became categorised as a level 3 herd. Therefore a trace back from that farm to
205 the supplier herd, farm C, was made and *S. Choleraesuis* was cultured in pen floor samples there in
206 August 2013. The farm had no known previous culture confirmed history of *Salmonella*. The farm,
207 which had purchased the 41 pigs from farm C, was not tested for *Salmonella* by culture, because the
208 owner decided to empty the farm soon after the diagnosis was made in farm C. The buildings were
209 then cleaned and disinfected before new pigs were installed. That farm has subsequently been tested
210 but *S. Choleraesuis* has not been found. Farm C is operating as before and with *S. Choleraesuis*
211 probably still present. Some management procedures have been changed to reduce infection and
212 contamination within herd and there have been repeated antibiotic treatments to reduce mortality.
213 Serological data of meat juice samples showed a very high sero-prevalence for some months
214 followed by a dramatic decrease, and after July 2014 – at least until November – no seropositive
215 samples have been detected.

216 Farm D purchases pigs for on-growing from a single sow herd, which supplies pigs to several
217 other herds. Neither the supplying sow herd nor any of the other herds receiving pigs from that
218 supplier herd are or have been tested positive with *S. Choleraesuis*. Farm D had experienced high
219 *Salmonella* sero-prevalence since October 2012 (Figure 2) and there was a general, very high
220 mortality, approximately 20 – 30 %, among 30 – 50 kg pigs. There was no known history of
221 *Salmonella*. In December 2013 dead pigs were submitted to a laboratory with an anamnesis of
222 diarrhoea, respiratory problems and septicaemia, and *S. Choleraesuis* was cultured from the pigs.
223 Clinical salmonellosis is subjected to antibiotic treatment, but mortality is still high and it is still

224 infected with *S. Choleraesuis*. Moderate to high proportions of sero-positive meat juice samples are
225 still detected in the herd (Figure 2)

226 Both farm C and D have purchased corn directly from Eastern Europe, delivered on site by
227 truck, without involving a feed company and without heat treatment.

228 The outbreak on farm E in 1999 has been described elsewhere (Baggesen *et al.*, 2000) and will
229 not be further dealt with here.

230

231 2.2. *Pulsed-field gel electrophoresis*

232 A total of five different PFGE patterns were recognized among the 23 isolates (Figure 3). The
233 14 isolates from the outbreak on farm E in 1999 - 2000 displayed three different profiles (containing
234 4, 9, and 1 isolate, respectively), but they clustered together as they deviated in only one or two
235 bands. The five isolates from the two farms A and B that were recovered from the outbreaks in
236 2012 - 2013, had identical PFGE profiles. These two farms were also epidemiologically connected,
237 i.e. farm A had delivered pigs to farm B, as described above. The three isolates from farm C and the
238 isolate from farm D recovered from outbreaks in 2013 were indistinguishable (Figure 3).

239

240 2.3. *Whole genome sequencing*

241 On the basis of the WGS data, all tested isolates were found to have MLST ST1804, a type
242 which has not previously been reported in the PubMLST database
243 (<http://mlst.ucc.ie/mlst/dbs/Senterica>). rMLST divided the isolates into two groups. The isolates

244 from 2012 – 2013 belonged to ST3723 and the isolates from 1999 to ST3636, differing by a single
245 nucleotide mismatch in one of the alleles (Figure 4).

246 A total of 672 SNPs were identified and used to construct a phylogenetic tree (Figure 4). None
247 of the Danish isolates from 1999 – 2000 and 2012 – 2013 were identical but clustered together in
248 two groups. The isolates from 1999 – 2000 had less than 93 SNPs difference between them, while
249 the isolates from 2012 – 2013 separated in two subgroups with 23 and 134 SNPs difference
250 between the isolates, respectively. The two isolates from farm A and B, which were
251 epidemiologically related, had only 23 SNPs difference, indicating a close relatedness. This was
252 underlined by the fact that they also had identical PFGE profiles. The two other isolates, farm C and
253 D, had more SNPs difference, indicating that they were less closely related, although they shared
254 the same PFGE profile but different from that of farm A and B.

255 To further characterize the isolates WGS data were analysed for the content of plasmid
256 replicons, and acquired antimicrobial resistance genes. All sequenced isolates contained plasmid
257 replicons of the *incFIB* and *incFII* type. The three isolates from 2012 – 2013 in addition, carried
258 plasmids of replicon type *incQ1*, while the isolates from 1999 had three different replicon types
259 (Figure 4).

260

261 2.4. *Antimicrobial susceptibility*

262 The phenotypic antimicrobial susceptibility of the seven isolates obtained by MIC determination
263 is shown in Figure 3. The four isolates from the outbreaks in 2012 – 2013 shared the same
264 resistance profile, being resistant to sulphonamides and streptomycin. The three isolates from 1999
265 had different resistance profiles: one was resistant only to streptomycin whereas the two others were

266 multi-resistant to streptomycin, spectinomycin, sulphonamides and tetracyclines, and one of them
267 additionally to trimethoprim (Figure 4).

268 The MIC results corresponded well for most of the isolates with the contents of antimicrobial
269 resistance genes, which were identified from the WGS data. No resistance genes were shared
270 between isolates from 1999 – 2000 and isolates from 2012 – 2013 (Figure 4).

271

272 3. Discussion

273 Most often pigs are symptomless carriers of Salmonella, although *S. Typhimurium* (including
274 monophasic variants) and occasionally other serovars including *S. Infantis* and *S. Derby* may cause
275 clinical salmonellosis with diarrhoea as primary clinical symptom. In contrast, diarrhoea is less
276 pronounced among pigs infected with *S. Choleraesuis*. Clinical symptoms here usually include
277 fever, inappetence, lethargy, and respiratory symptoms with coughing and difficulties breathing.
278 Symptoms often appear 24 – 36 hours post infection, and gastrointestinal symptoms usually appear
279 after 4 – 5 days (Fedorka-Cray *et al.* 2000). *S. Choleraesuis* may be dormant in a pig farm but then
280 be activated by stress factors, such as porcine circovirus (PCV2) or porcine reproductive and
281 respiratory syndrome (PRRS) virus outbreaks (Chiu *et al.*, 2004). In the Danish herds in 2012 –
282 2013 there was a concurrent outbreak of oedema disease in farm A, and both farm C and D had
283 several concurrent disease problems with high mortality. It can only be suggested whether this has
284 contributed to the severity of the *S. Choleraesuis* outbreaks in these herds or vice versa, but the
285 occurrence of other diseases at the same time has likely delayed the diagnosis of *S. Choleraesuis*,
286 because the herds were treated for the infections that were known to be present, and for a long time
287 no further laboratory investigation was made to look for other pathogens. Huang *et al.* (2009) found
288 high levels of resistance to ampicillin, tetracycline and ticarcillin, but low resistance to

289 spectinomycin and no resistance to other tested compounds in a collection of American isolates. In
290 general, from European countries and the USA, there are not many reports on antimicrobial
291 susceptibility of this serovar. In the present study, we fortunately found only low levels of
292 resistance, i.e. the isolates from 2012 – 2013 were only resistant to streptomycin and sulfonamides,
293 while the isolates from the 1999 outbreak were resistant to more compounds. Notably, critical
294 resistance to fluoroquinolones or cephalosporins was not found.

295 The source of the infection in the Danish herds is presently unknown. Apart from the trade of
296 pigs from farm A to B, there are no common factors that connect them, and they are located
297 geographically distant from each other (Figure 1). It is generally accepted that *S. Choleraesuis* is
298 rarely found in feed or in animals other than pigs, and that the source is consequently most often
299 limited to horizontal transfer by carrier pigs (Anderson *et al.* 2000). Experimental infection studies
300 in two days old pigs have shown that some of the pigs were shedding the bacterium for at least 85
301 days (Anderson *et al.* 2000), during which period they are potentially able to transfer the infection,
302 which is readily transmitted to uninfected pigs via contact with infected animals or their faeces
303 (Gray *et al.* 1996). In the present cases from 2012 – 2013, no pigs were imported into three of the
304 herds, which rules out carrier pigs as source in these herds. One herd (farm B) had received pigs
305 from one of the other herds (farm A), and since the PFGE patterns of isolates from these two
306 connected herds were identical and the SNP tree also showed close relatedness, it is concluded that
307 the infection was transferred from one herd to the other. Farm A is a new and well managed SPF
308 herd with a strict biosecurity and no entry of animals. It has not been possible to identify any
309 potential sources or routes of infection. The isolates from Farm C and D had indistinguishable
310 PFGE patterns and both farms had very poor biosecurity. Farm C had not received any pigs from
311 other farms, and farm D had only purchased pigs from a single supplier, which was not detected
312 with *S. Choleraesuis* infection. Therefore, it is concluded that live pigs were not the source of

313 infection in any of the herds, except for farm B. *S. Choleraesuis* has been found in wild boars in
314 Europe, at least in Italy (Chiari *et al.*, 2013; Zottola *et al.*, 2013) and Spain (Perez *et al.*, 1999),
315 which suggests a wildlife reservoir that may spill over to farmed pigs or vice versa. In Denmark
316 there is not a stock of wild boars so, although wild boars from Germany occasionally cross the
317 border, this source of infection can probably be ruled out.

318 Although the analyses here do not address where the infections came from, the typing data
319 together with the epidemiological information from the farms suggest that the outbreaks in 2012 –
320 2013 may have been caused by two or maybe three separate introductions rather than a single
321 event. This theory is supported by the fact that two different PFGE profiles were involved, and that
322 the SNP analysis grouped the isolates in two groups. On the other hand, all isolates had identical
323 MST types, resistance profiles, plasmid replicons and rMLST profiles, indicating that they were
324 closely related. At the outbreak in 1999 – 2000, three different PFGE profiles were found on the
325 same farm, which indicates some variability within this serovar. It therefore seems likely that there
326 was some kind of connection between the outbreaks although epidemiological investigations have
327 not been able to point out any common factors. It can only be speculated how the infection was
328 brought to the country. There is a considerable export of live pigs from Denmark to especially
329 Germany but also other European countries, and it is possible that the trucks returning to Denmark
330 may occasionally not have been properly cleaned and disinfected. Two of the farms had imported
331 corn directly delivered by truck from an area of Europe where *S. Choleraesuis* is endemic. Feed is
332 known occasionally to be contaminated with *Salmonella*, and may be a risk factor for introduction
333 of a plethora of *Salmonella* serovars into animal herds (Hald *et al.* 2012), but to the authors'
334 knowledge, *S. Choleraesuis* has never been found in animal feed in Denmark. Yet, it cannot be
335 entirely ruled out that such feed shipments may have been contaminated with the bacterium.
336 However, the four farms all had different suppliers of feed, so it can be excluded that there was a

337 common source of entry from feed. *Salmonella* survives well in the environment. Although *S.*
338 *Choleraesuis* is host adapted and often believed not to survive well outside a host, experiments have
339 shown that it is able to survive in faeces from infected pigs for at least 13 months and be infective
340 for at least 4 months (Gray and Fedorka-Cray 2001). Therefore, hygiene and biosecurity measures
341 are extremely important for prevention of *Salmonella* in pigs and care must be taken to clean and
342 disinfect equipment, etc., and not allow faecal contaminated equipment to enter. Care must in
343 particular be taken when pigs are collected by trucks for sale or slaughter, or when trucks deliver
344 feed to the farm that no faecal material or contaminated equipment from the truck is introduced.

345 Although humans have not been described to be carriers of *S. Choleraesuis*, humans have been
346 implicated as passive vectors, contributing to the spread between animals and herds (Wolf *et al.*,
347 2011). In the present cases there is no evidence that humans have been the source or vector of the
348 infection, as three of the farms had no foreign employees.

349 In conclusion: *S. Choleraesuis* was reintroduced in four Danish pig herds in 2012 – 2013 after
350 12 years absence, and increasing meat juice sero-prevalence against *Salmonella* was detected in the
351 herds prior to isolation of *S. Choleraesuis*. In spite of intensive molecular typing, the sources of
352 infection could not be traced on the basis of the current investigation. The infections were likely not
353 brought by live animals or humans but direct imported feed cannot be excluded as source. The
354 results suggest that it was two or three independent introductions but typing data indicated that the
355 isolates were related and ongoing WGS on a larger collection of *S. Choleraesuis* from many
356 countries may allow better conclusions on potential sources to be drawn.

357

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362

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483

484 **Figure legends**

485

486 Figure 1. Approximate location of the Danish farms infected with *S. Choleraesuis* and general farm
487 information. Data for farm E have been described elsewhere (Baggesen *et al.* 2000).

488

489 Figure 2. Data for *Salmonella* sero-prevalences on the four outbreak farms together with detection
490 of *Salmonella* Choleraesuis or other serovars. Data for the period January 2010 – November 2014
491 are included.

492

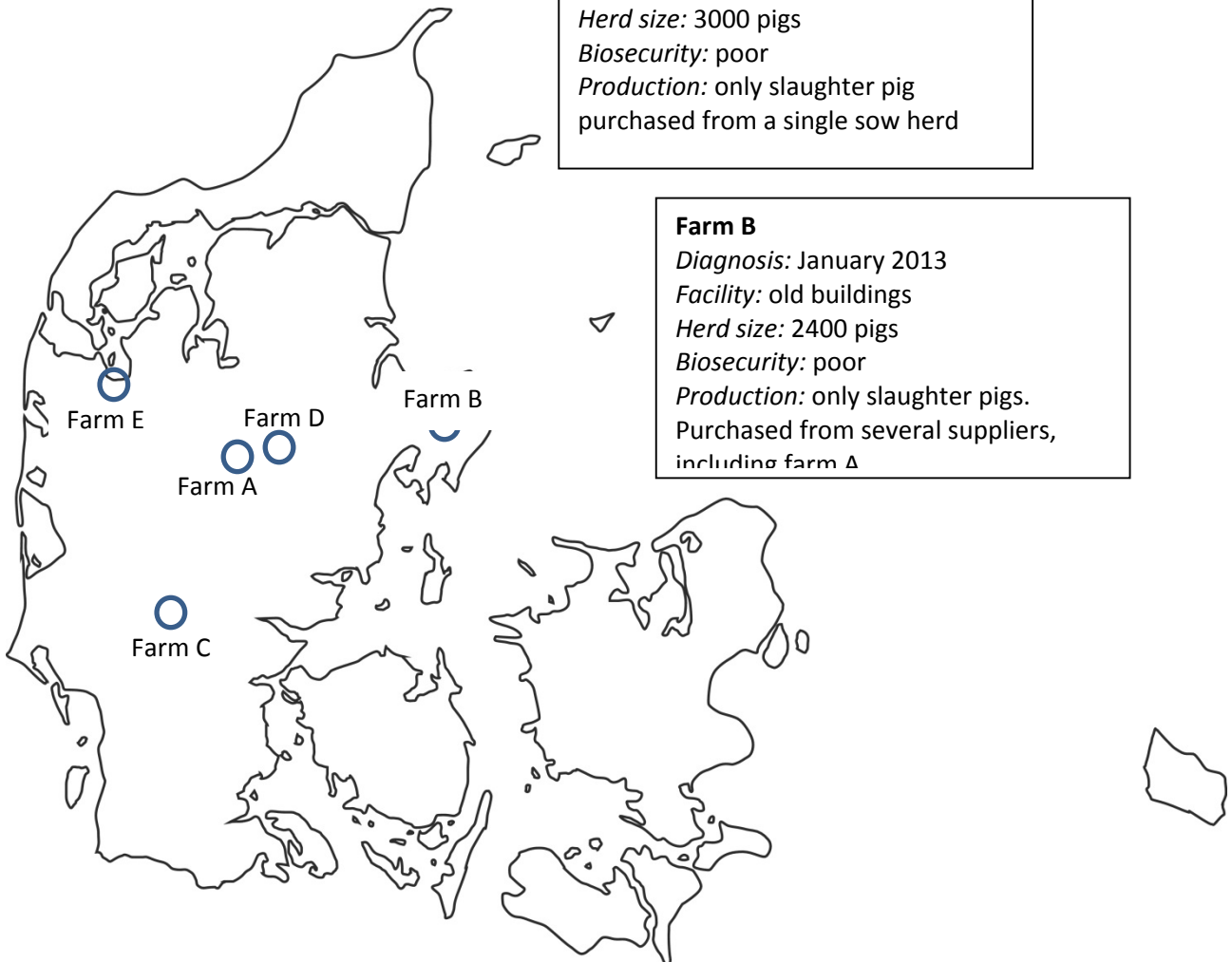
493 Figure 3. Dendrogram of 23 Danish isolates of *S. Choleraesuis* from five pig farm outbreaks
494 produced from pulsed-field gel electrophoresis results. Isolates marked with an asterisk were
495 subjected to whole genome sequencing.

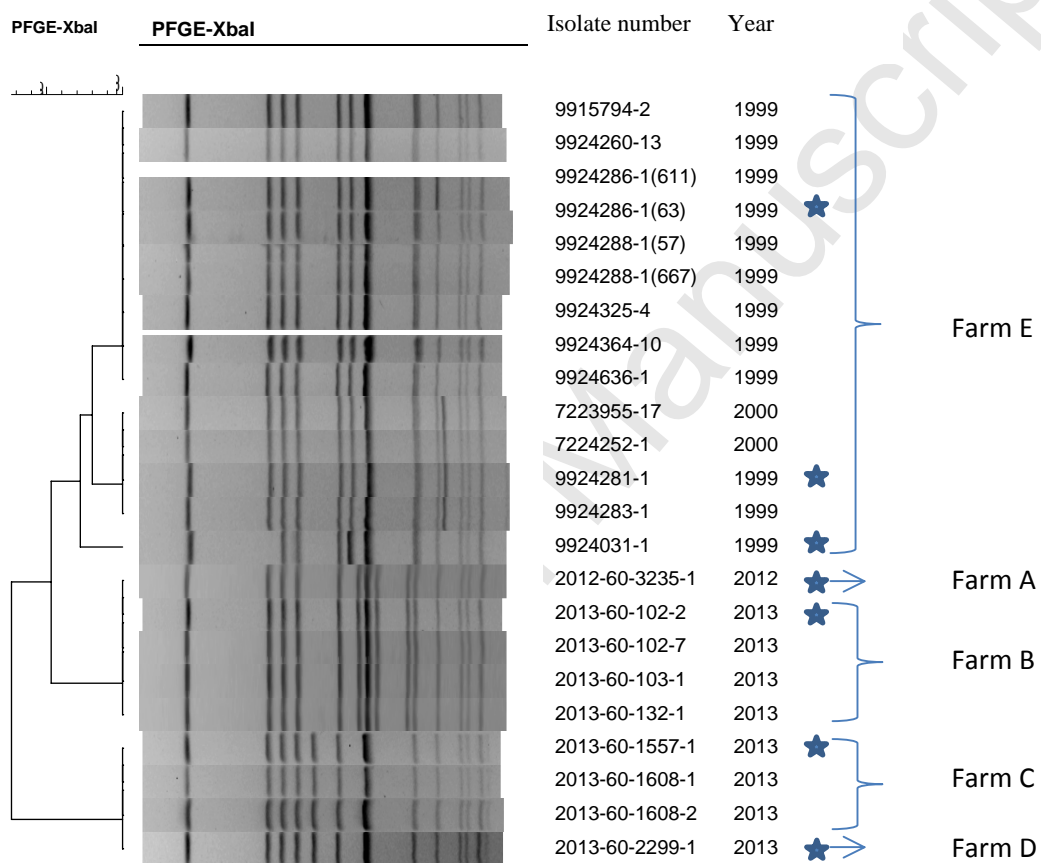
496

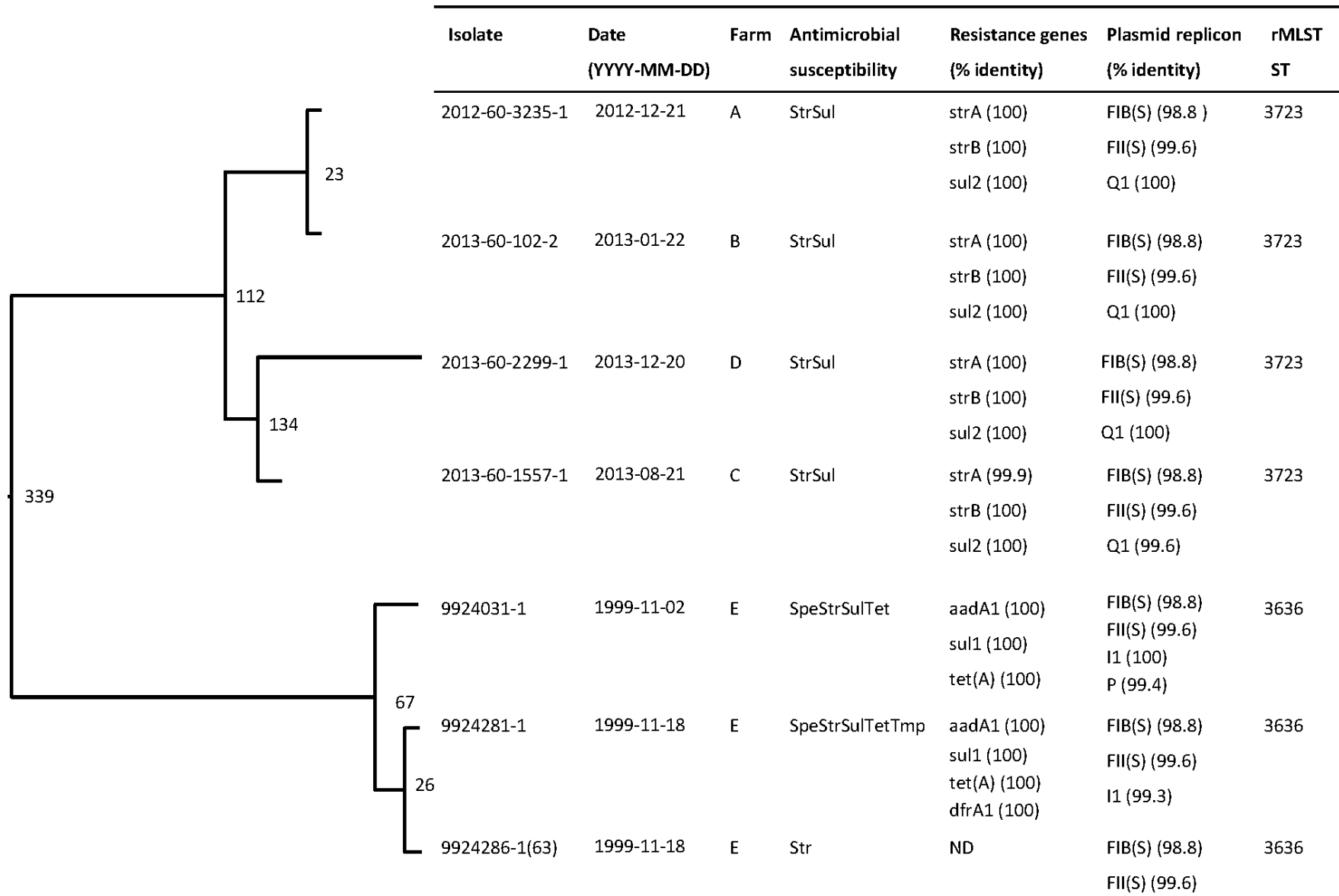
497

498 Figure 4. SNP tree together with results for the antimicrobial susceptibility tests, presence of
499 antimicrobial resistance genes and plasmids for the seven sequenced isolates of *S. Choleraesuis*
500 from Danish pig farms. SNP differences between branches are indicated with numbers in the
501 dendrogram. The total no. of SNPs was 672. Str = streptomycin, Sul = sulfonamides, Spe =
502 spectinomycin, Tet = tetracyclines, Tmp = trimethoprim. ND = none detected, ST = sequence type.

503

Farm A*Diagnosis:* December 2012*Facility:* modern, built 2008*Herd size:* 5250 pigs, including 650 sows*Biosecurity:* high. Specific pathogen free (SPF) herd*Production:* produces slaughter pigs and sells pigs for on-growing and export. No pigs are bought to the farm**Farm D***Diagnosis:* December 2013*Facility:* old buildings*Herd size:* 3000 pigs*Biosecurity:* poor*Production:* only slaughter pig purchased from a single sow herd**Farm B***Diagnosis:* January 2013*Facility:* old buildings*Herd size:* 2400 pigs*Biosecurity:* poor*Production:* only slaughter pigs. Purchased from several suppliers, including farm A**Farm C***Diagnosis:* August 2013*Facility:* old buildings*Herd size:* 2410 pigs, including 220 sows*Biosecurity:* very poor*Production:* produces slaughter pigs and sells pigs for on-growing. No pigs





Highlights

- Salmonella Choleraesuis reappeared in Danish pig herds in 2012 – 2013
- Outbreaks were preceded by increased meat juice sero-prevalence
- Severe disease problems occurred in affected herds
- Two or three independent introductions occurred based on molecular typing and epidemiology
- Sources of the infection could not be established

