

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

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

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19

20 Abstract

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

43 Introduction

Pork is one of the most important sources of human foodborne salmonellosis in the EU (EFSA, 44 45 2013) and the USA (Gould et al., 2013). Pigs can be colonized with a variety of Salmonella serotypes (EFSA 2008, 2009) but mostly, pigs are asymptomatic carriers. In Denmark, the most 46 47 common Salmonella serovars in pigs are Salmonella enterica serovar Typhimurium (S. Typhimurium) (including monophasic S. 4, [5], 12:i:-), S. Derby and S. Infantis (Argüello et al., 48 2013, 2014). These serovars may also cause clinical salmonellosis in pigs, but the extent of clinical 49 salmonellosis in pigs in Denmark is uncertain. 50 S. Choleraesuis is a serovar, which is host-adapted to pigs, and may cause serious outbreaks of 51 salmonellosis and paratyphoid (Griffith et al. 2006). The majority of the S. Choleraesuis outbreaks 52

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

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

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

68 In humans, S. Choleraesuis tends to be more invasive and cause less gastrointestinal manifestations than most other serotypes and thus, it is a serious infection with a significant 69 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 countries, such as Thailand and Taiwan, this serovar continues to be important for human illness 75 (Chiu et al., 2004; Hendriksen, 2010), although the incidences seem to be declining (Su et al., 76 2014). 77

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

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

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

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

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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 and112 Weill, 2007).

113

114 *1.3. Pulsed-field gel electrophoresis*

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

121

122 *1.4.* Antimicrobial

Antimicrobial resistance profile

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

132 *1.5. W*

133

Whole genome sequencing, multilocus sequence typing, antimicrobial resistance genes, plasmid replicons, and plasmid multilocus sequence typing

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

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

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

153

154 *1.6.* Single Nucleotide Polymorphisms

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

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

B, C, and D. In addition, isolates for the investigations from an outbreak in 1999 on a farm,

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

180 Farm A experienced a dramatic increase in Salmonella sero-prevalence in meat juice samples in August 2012 and became classified as a level 2 herd in September and level 3 in October (Fig 2) 181 Concomitantly, there was an outbreak of oedema disease, and the mortality increased to appr. 20 % 182 183 among pigs up to 30 kg. Antibiotic treatment was initiated at this point, but unfortunately, no samples were taken for *Salmonella* culture. The farm had previously in January 2011 been 184 diagnosed with S. Infantis. It was not until November 2012 that carcasses were submitted to a 185 186 laboratory and S. Choleraesuis was identified from lung tissue. The pigs were subsequently culled and the premises cleaned and disinfected, and in February 2013 new SPF animals were installed. 187 Pen floor samples collected in January 2014 to follow up on Salmonella status did not show 188 presence of S. Choleraesuis, but S. Infantis was still present. Serological data showed that the herd 189 continued to have a low proportion of sero-positive samples (Figure 2) – possibly due to S. Infantis. 190 Farm B purchased pigs from farm A during the summer 2012, and when farm A was diagnosed 191 with S. Choleraesuis, farm B was also investigated due to the registered transfer of pig. At that time, 192 the Salmonela seroprevalence in the herd had changed from zero to low and the farm had 193 experienced increased mortality, and upon laboratory investigation of dead pigs, the farm was 194 195 diagnosed with S. Choleraesuis as well as S. Derby in January 2013. The herd was then culled; some of the oldest buildings were destroyed, and the remaining were cleaned and disinfected, where 196 after the farm started to operate again. Follow-up pen floor samples collected in September 2013 197 did not show presence of S. Choleraesuis but both S. Derby and S. Infantis were found. Serological 198 data showed that a moderate proportion of sero-positive samples was still present (Figure 2). 199

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

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

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

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

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

230

231 2.2. Pulsed-field gel electrophoresis

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

239

240 2.3. Whole genome sequencing

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 (<u>http://mlst.ucc.ie/mlst/dbs/Senterica</u>). rMLST divided the isolates into two groups. The isolates

from 2012 – 2013 belonged to ST3723 and the isolates from 1999 to ST3636, differing by a single
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 of the Danish isolates from 1999 - 2000 and 2012 - 2013 were identical but clustered together in 247 two groups. The isolates from 1999 – 2000 had less than 93 SNPs difference between them, while 248 the isolates from 2012 – 2013 separated in two subgroups with 23 and 134 SNPs difference 249 250 between the isolates, respectively. The two isolates from farm A and B, which were epidemiologically related, had only 23 SNPs difference, indicating a close relatedness. This was 251 252 underlined by the fact that they also had identical PFGE profiles. The two other isolates, farm C and D, had more SNPs difference, indicating that they were less closely related, although they shared 253 the same PFGE profile but different from that of farm A and B. 254

To further characterize the isolates WGS data were analysed for the content of plasmid replicons, and acquired antimicrobial resistance genes. All sequenced isolates contained plasmid replicons of the *inc*FIB and *inc*FII type. The three isolates from 2012 – 2013 in addition, carried plasmids of replicon type *inc*Q1, while the isolates from 1999 had three different replicon types (Figure 4).

260

261 2.4. Antimicrobial susceptibility

The phenotypic antimicrobial susceptibility of the seven isolates obtained by MIC determination is shown in Figure 3. The four isolates from the outbreaks in 2012 – 2013 shared the same resistance profile, being resistant to sulphonamides and streptomycin. The three isolates from 1999 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 them267 additionally to trimethoprim (Figure 4).

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

271

3. Discussion

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

spectinomycin and no resistance to other tested compounds in a collection of American isolates. In
general, from European countries and the USA, there are not many reports on antimicrobial
susceptibility of this serovar. In the present study, we fortunately found only low levels of
resistance, i.e. the isolates from 2012 – 2013 were only resistant to streptomycin and sulfonamides,
while the isolates from the 1999 outbreak were resistant to more compounds. Notably, critical
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 pigs from farm A to B, there are no common factors that connect them, and they are located 296 297 geographically distant from each other (Figure 1). It is generally accepted that S. Choleraesuis is rarely found in feed or in animals other than pigs, and that the source is consequently most often 298 limited to horizontal transfer by carrier pigs (Anderson et al. 2000). Experimental infection studies 299 in two days old pigs have shown that some of the pigs were shedding the bacterium for at least 85 300 days (Anderson et al. 2000), during which period they are potentially able to transfer the infection, 301 302 which is readily transmitted to uninfected pigs via contact with infected animals or their faeces (Gray *et al.* 1996). In the present cases from 2012 - 2013, no pigs were imported into three of the 303 herds, which rules out carrier pigs as source in these herds. One herd (farm B) had received pigs 304 305 from one of the other herds (farm A), and since the PFGE patterns of isolates from these two connected herds were identical and the SNP tree also showed close relatedness, it is concluded that 306 307 the infection was transferred from one herd to the other. Farm A is a new and well managed SPF herd with a strict biosecurity and no entry of animals. It has not been possible to identify any 308 potential sources or routes of infection. The isolates from Farm C and D had indistinguishable 309 310 PFGE patterns and both farms had very poor biosecurity. Farm C had not received any pigs from other farms, and farm D had only purchased pigs from a single supplier, which was not detected 311 with S. Choleraesuis infection. Therefore, it is concluded that live pigs were not the source of 312

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

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

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

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

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

357

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- 482 483

484 Figure legends

485

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

488

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

492

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

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







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





