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Insight into an outbreak of *Salmonella* Choleraesuis var. Kunzendorf in wild boars

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ABSTRACT

An unusual mortality of wild boars occurred in Italy from 2012 to 2015 due to *Salmonella* Choleraesuis infection.

In order to confirm the occurrence of an outbreak of *S.* Choleraesuis in wild boars and to epidemically characterise the unique *S.* Choleraesuis biovar, a collection of isolates belonging to wild boars was investigated from the phenotypic, molecular and genomic points of view (PFGE and WGS). Moreover, the possibility of transmission to domestic pigs and humans, temporally and geographically close to the wild boar epidemic, was tested by also including in the panel isolates from infected domestic pigs and from one human case of infection.

Wild boar isolates displayed a high genetic correlation, thus suggesting they are part of the same outbreak, with a common invasiveness potential. Conversely, no correlation between pig isolates and those from the other sources (wild boars and human) was found. However, the phylogenetic and PFGE analyses suggest a high degree of similarity between the human and the investigated wild boar outbreak isolates, implying the potential for the spread of *Salmonella* Choleraesuis among these species.

1. Introduction

Salmonella enterica, a common zoonotic pathogen, consists of over 2500 known serovars. Among these, *S.* Choleraesuis, a porcine-adapted patvhogen can cause serious outbreaks of salmonellosis and paratyphoid in pigs (Chiu et al., 2004; Pedersen et al., 2015) often resulting in fatal systemic disease (Boyen et al., 2008). The pathogen can lie dormant in herds until activated by external stressors (Chiu et al., 2004) such as porcine circovirus (PCV2) or porcine reproductive and respiratory syndrome (PRRS) virus co-infection (Pedersen et al., 2015). Nevertheless, *S.* Choleraesuis is able to survive in faeces from infected swine for a long time (Pedersen et al., 2015) and remains infective in the environment. The shedding of the organism by infected animals can result in long-term environmental contamination and continued re-infection of other animals. Furthermore, contaminated environments can occasionally serve as reservoirs of *S.* Choleraesuis also for humans, causing severe and invasive infections (Chiu et al., 2004).

S. Choleraesuis was the predominant serovar isolated from pigs worldwide during the 1950s and 1960s. Nevertheless, this serovar is currently highly prevalent in North America and Asia (Luk-in et al., 2018), but is rare in Australia and the European Union (EU) (Fedorka-Cray et al., 2000). In Europe, several cases of outbreak of the septicaemic form, caused by *S.* Choleraesuis var. Kunzendorf, were reported in wild boars: for example in Spain in 1999 (Pérez et al., 1999); in Thuringia between 2006 and 2008 (Methner et al., 2010); in central-western Spain between 2010 and 2016 (Gil Molino et al., 2019), and; in the German federal state North Rhine-Westphalia in 2017 (Methner et al., 2018).

Despite the low prevalence in pigs, *S.* Choleraesuis is becoming more prevalent in wild boars in Europe (Gil Molino et al., 2019) due to population increases reported during the last decades (Massei et al., 2015). This demographic explosion could result in increased exposure of humans to wild swine zoonotic pathogens, thus enhancing the chances of disease transmission (Gil Molino et al., 2019). This could be due to direct contact with wild environments through human settlements in semi-natural areas, out-door activities (Ruiz-Fons, 2017), and *via* food, especially in the case of game meat consumption. Moreover, wild boars can also play a prominent role in the exchange of zoonotic

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pathogens with livestock environments (Gil Molino et al., 2019), possibly leading to dramatic socio-economic impact as in the case of swine fever (Cabezón et al., 2017).

An unexpected and sudden occurrence of a severe epizootic of *S.* Choleraesuis in Italy (Conedera et al., 2014), from February 2012 until June 2015, raised some concerns for both human health and pig production. For this reason, passive surveillance was promptly increased, allowing the health authorities to collect 83 wild boars found dead in the field, most of which showed pathological lesions of septicaemia. Active surveillance was also implemented in regularly hunted wild boars, leading to the collection of animal samples from 345 hunted animals. A significant association between disease-death and infection by *S.* Choleraesuis was found (Conedera et al., 2014).

In order to definitely confirm the outbreak of *S.* Choleraesuis in wild boars and epidemically characterise *S.* Choleraesuis unique strains and determine possible transmission to pigs and humans, we studied, from the phenotypic, molecular and genomic points of view, a collection of isolates from wild boars (both septicaemic and hunted) and compared them with isolates belonging to infected pigs and a human case that occurred in the same geographic area and timeframe.

2. Methods

2.1. Bacterial isolates

A total of 79 isolates were analysed in this work. All the isolates were recovered according to ISO 6579-1:2017. A total of 58 isolates belonged to septicaemic wild boars found dead or those showing clinical disease in the field, while 18 isolates were from wild boars regularly culled in the context of hunting, displaying no clinical signs (although in some cases lesions were observed at slaughter). Salmonella isolates from wild boars were collected over the period 2012–2015. In addition, one isolate from a hospitalised female patient and two isolates from septicaemic domestic pigs farmed in a geographic region contiguous with the outbreak were included in the study. The geographic distribution of the isolates is represented in Fig. 1. The panel of analysed Salmonella isolates is described in Supplementary_Table_1.

2.2. Phenotypic serotyping and biochemical tests

All *Salmonella* isolates were serotyped according to the White–Kauffmann–Le Minor scheme (Grimont and Weill, 2008) by slide agglutination with polyclonal antisera (Statens Serum Institut, Copenhagen, Denmark), and distinction between the biovars of *S.* Choleraesuis, var. Kunzendorf and var. Decatur was performed by biochemical tests (H_2S production, mucate and dulcitol fermentation) (Grimont and Weill, 2008).

2.3. Molecular serotyping

Salmonella isolates showing incomplete antigenic formula according to slide agglutination serotyping were tested by xMAP® *Salmonella* Serotyping Assay (SSA; Luminex Corp., Austin, TX, U.S.) to address a set of target genes involved in the expression of the most common *Salmonella* serotype-specific antigens (Fitzgerald et al., 2007; Dunbar et al., 2014).

2.4. Pulsed-field gel electrophoresis (PFGE)

All *Salmonella* isolates were characterised by PFGE, which was carried out after digestion of genomic DNA with the restriction enzyme *Xba*I (Jacobs et al., 2014). Gel images were analysed by BioNumerics version 7.6 (Applied Maths, Sint-Martens-Latem, Belgium) and compared by cluster analysis using the Dice coefficient for similarity and the unweighted pair group method with arithmetic means (UPGMA), with a position tolerance of 2% and optimisation of 1%.

2.5. Isolate selection for genomic analysis

Wild boar isolates (n = 30) were chosen for whole genome sequencing (WGS) using a spatial distribution criterion by applying the simple random sampling tool in the open source software QGis (Tethys 2.18 - www.qgis.org) to a geo-database including all 76 wild boar isolates. To investigate genetic features responsible for S. Choleraesuis virulence and invasiveness, wild boar isolates were sampled from both septicaemic (found dead, n = 15) and hunted (asymptomatic, n = 15) animals. The infected human (n = 1) and infected domestic pig isolates (n = 2) temporally and spatially close to the outbreak were also characterised by WGS.

2.6. Whole genome sequencing analyses

Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and quantified with a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA). Libraries for sequencing were prepared using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). High-throughput sequencing was performed on Illumina MiSeq with 2 × 250 paired-end reads.

Raw sequence data were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession number PRJEB27935. Raw reads were assembled using SPAdes (version 3.9) (Nurk et al., 2013), available at the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org).

Assembled sequences were analysed to identify species, MLST sequence type (ST) for *Salmonella enterica*, plasmid replicons, antimicrobial resistance genes and presence of *Salmonella* Pathogenicity Islands (SPI) using the tools available on the CGE website KmerFinder (version 2.0) (Hasman et al., 2014; Larsen et al., 2014), MLST (version 1.8) (Larsen et al., 2012), PlasmidFinder (version 1.3) (Carattoli et al., 2014), ResFinder (version 3.0) (Zankari et al., 2012) and SPIFinder (version 1.0).

The tool MyDbFinder 1.1 was used to detect virulence genes or plasmid sequences, i.e., pSCV50 (AB040415.1) virulence plasmid typical of *S.* Choleraesuis, its genes *spvR* (AB040415.1:1161-2054), *spvA* (AB040415.1:2565-3332), *spvB* (AB040415.1:3515-5290), *spvC* (AB040415.1:5571-6296), *spvD* (AB040415.1:6556-7206) and the toxin/antitoxin *ccdB/ccdA* (AY509003.1:12831-13136/13138-13356) genes.

Serotyping at the genetic level was performed analysing contigs with SeqSero (version 1.2) (Zhang et al., 2015) and raw reads with Salmonella TypeFinder (version 1.4) (Inouye et al., 2014; Zhang et al., 2015; Ashton et al., 2016).

A SNP-based phylogenetic tree was built using CSIPhylogeny (version 1.4) (Kaas et al., 2014) using *S.* Choleraesuis strain SC-B67 (AE017220) as the reference genome. The qualified SNPs were selected using the following criteria: a minimum coverage of 10, a minimum distance of 10 bp between each SNP and a minimum quality score for each SNP of 30.

3. Results

3.1. Isolate serotyping

A total of 64 isolates, including pig and human isolates, were found to be *S.* Choleraesuis var. Kunzendorf by phenotypic serotyping and biochemical tests, i.e. were able to express both the somatic and flagellar antigens, while 15 isolates displayed incomplete antigenic formulae, and thus, no definitive serovar was assigned to them (Supplementary_Table_1) (Figs. 2 and 3). *Salmonella* isolates showing incomplete antigenic formula were analysed by xMAP® *Salmonella* Serotyping Assay (SSA). Six isolates presented a complete antigenic formula corresponding to the serovar Choleraesuis (C1:c:1,5), while for the other nine isolates, somatic antigen sequences were not detected

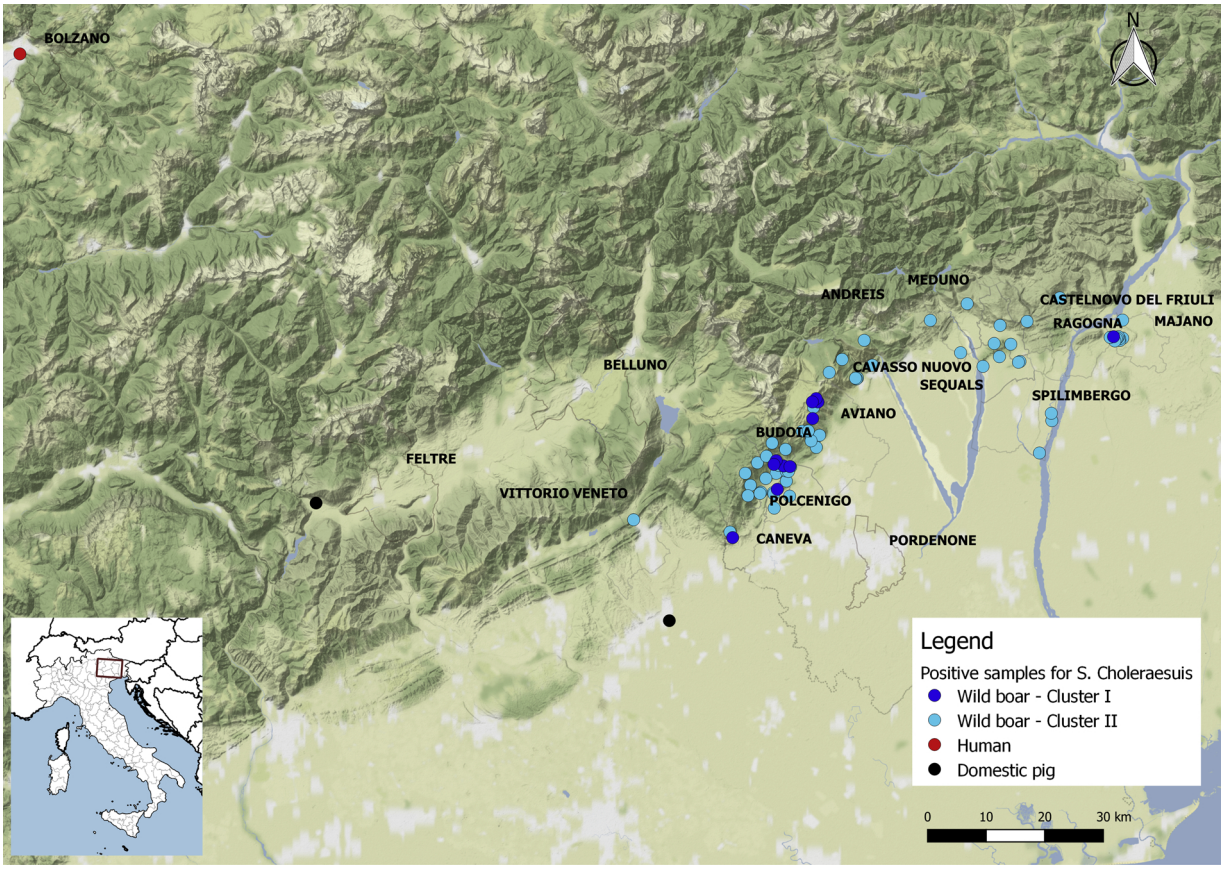

Fig. 1. Geographic distribution of the analysed isolates. Software used: QGis (Tethys 2.18 - www.qgis.org).

(Supplementary_Table_1).

Looking at the DNA sequences obtained by WGS, all tested isolates were clustered as *Salmonella enterica* species, serovar Choleraesuis var. Kunzendorf, Sequence Type 145, with the exception of five isolates that did not present a somatic antigen sequence (Fig. 4).

3.2. PFGE analysis

Three different pulsotypes, namely cluster I, II and III, were recognised among the whole panel of isolates. The pulsotypes I and II were closely related with a genetic similarity index of 92.3%, while the pulsotype III presented a genetic similarity index of 86.4% with clusters I and II (Fig. 2). Most of the isolates belonged to pulsotype II (66/79; 83.5%), 11 to pulsotype I (11/79; 13.9%) and 2 to pulsotype III (2/79; 2.5%). Most of the isolates from septicaemic animals (55/58, 95%), 10 out of 18 isolates from hunted animals (10/18, 55.5%) and the human isolate belonged to pulsotype II cluster, while the two domestic pig isolates belonged to pulsotype III. A graphical description of sample clustering according to pulsotype is reported in Fig. 3 and data are given in Supplementary_Table_1.

3.3. Genotypic analisis

3.3.1. Genotypic characterisation of antibiotic resistance

No antibiotic resistance genes were found in human and wild boar isolates. Conversely, domestic pig isolates displayed a panel of

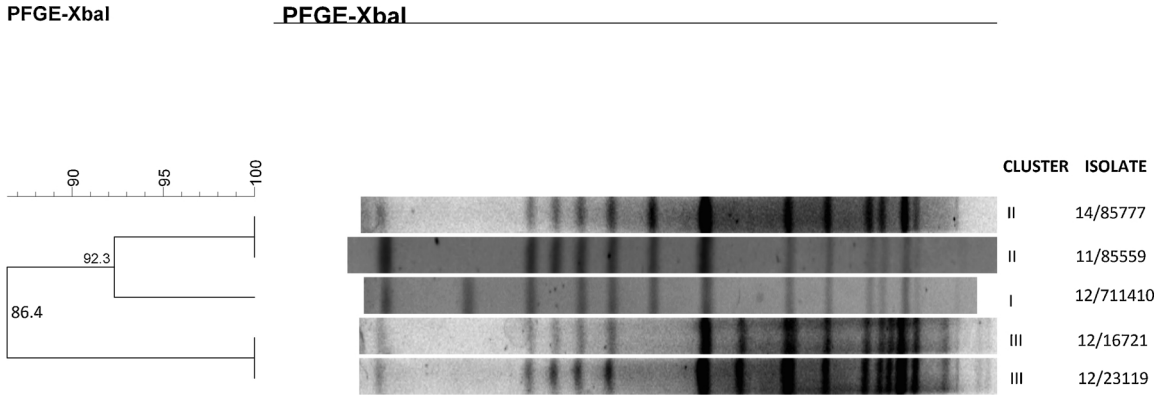

Fig. 2. Comparison of the three PFGE clusters. PFGE banding patterns of XbaI-digested chromosomal DNA of five isolates are reported as example of the three identified clusters. Images are derived by three different gels reported in Supplementary_Figure_1. Isolate 11/85559 derives from gel 1; isolate 12/171410 from gel 2 and isolates 14/85777, 12/16721 and 12/23119 from gel 3. Image analysis was conducted using BioNumerics version 7.6 software.

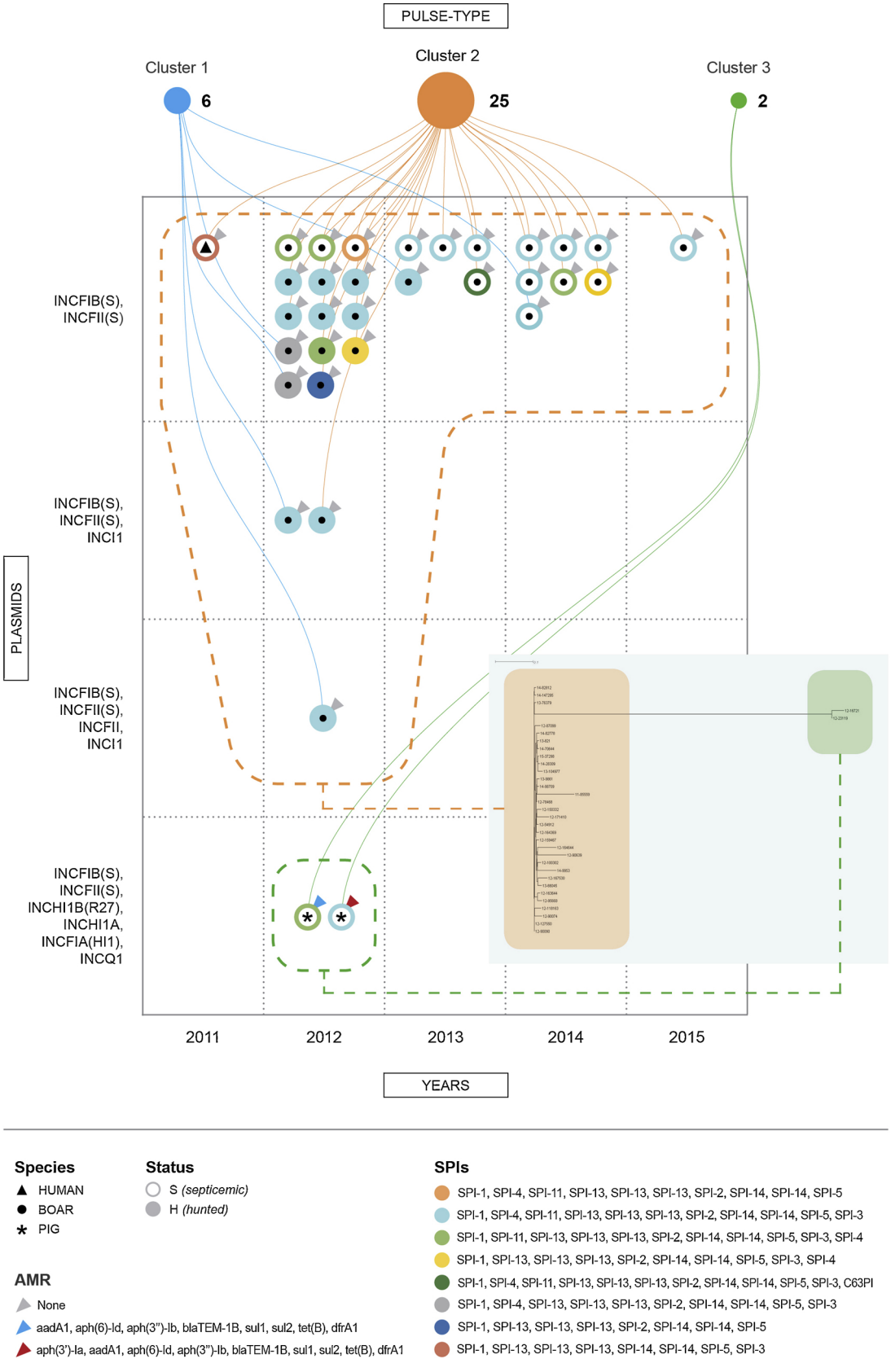

Fig. 3. Summary infographic of the 33 sequenced isolates. Information about species, status, year of isolation, plasmid replicons, AMR and SPI profile, pulse-type and phylogenetic analysis of the isolates are reported.

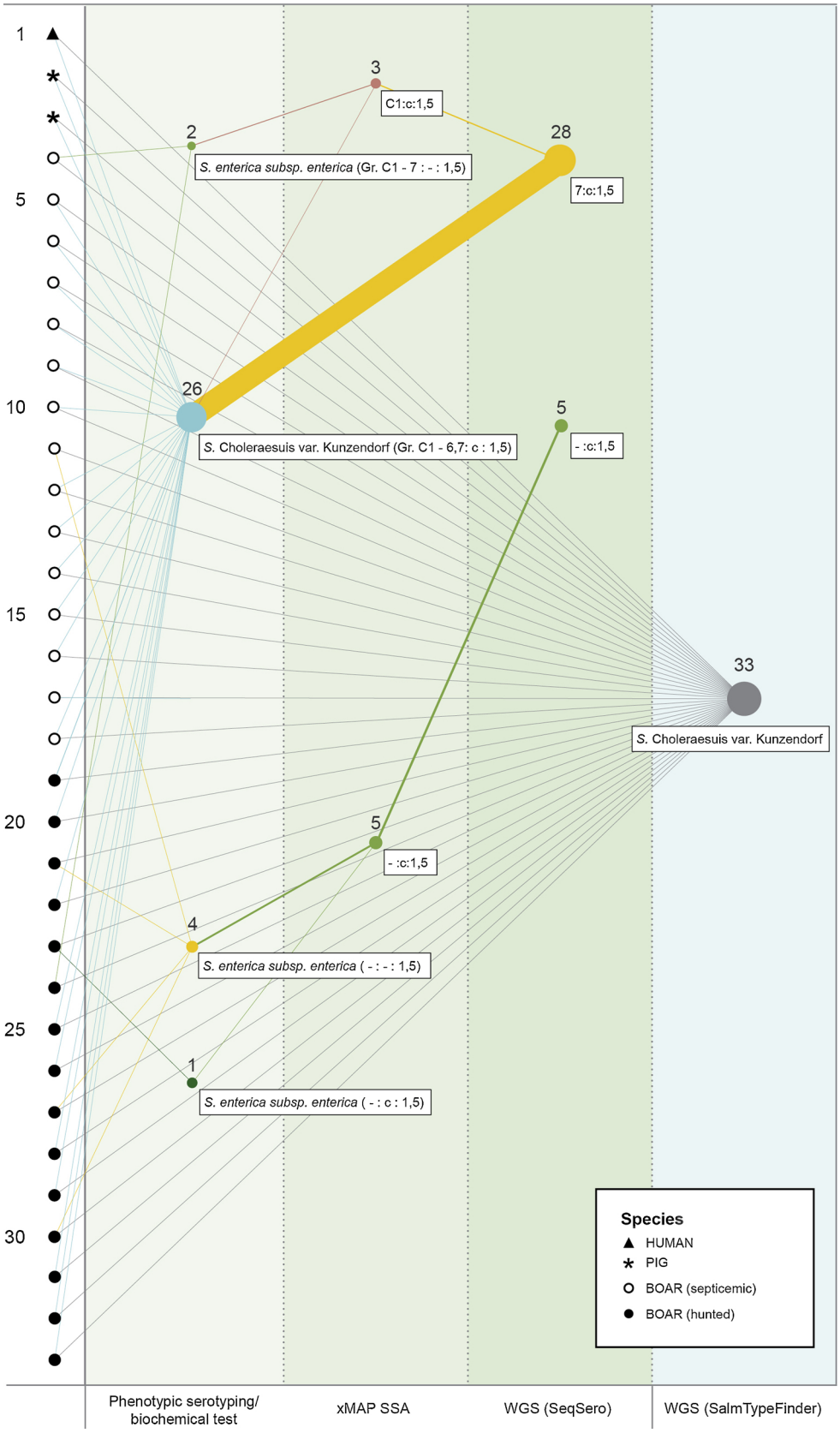

Fig. 4. Serotyping infographic of the 33 sequenced isolates using four methods: phenotypic serotyping and biochemical tests, xMAP® *Salmonella* Serotyping Assay (SSA), WGS (data analysis with SeqSero tool) and WGS (data analysis with SalmonellaTypeFinder tool). A total of 26 isolates out of 33 were found to be *S.* Choleraesuis var. Kunzendorf by phenotypic serotyping and biochemical tests, while 7 isolates displayed incomplete antigenic formulae: 7:-:1,5 (2), -:c:1,5 (1) and -:-:1,5 (4). The xMAP® SSA was performed on 8 isolates: three of them presented a complete antigenic formula (C1:c:1,5), but the other 5 did not display the somatic antigen sequence. WGS and data analysis with SeqSero tool allowed recognition of 28 complete antigenic formulae (7:c:1,5) and confirmed the absence of somatic antigen sequences already observed with xMAP® SSA. WGS and data analysis with SalmonellaTypeFinder tool permitted clustering of all 33 sequenced isolates as *S.* Choleraesuis var. Kunzendorf.

antibiotic resistance genes that conferred resistance to aminoglycosides, beta-lactams, sulphonamides, tetracyclines and trimethoprim. Information about antibiotic resistance genes is reported in Fig. 3.

3.3.2. *Plasmid typing*

All sequenced isolates contained plasmid replicons belonging to IncFIB and IncFII types; these replicons were found to be co-localised on the same contig. In addition, pig isolates displayed IncHI1 and IncQ1

replicons, as reported in Fig. 2. In particular, IncQ1 replicon was found on the same contig together with *aadA1*, *aph (6) -Id*, *aph (3'')-Ib*, *sul2*, *sul1* and *dfrA1* resistance genes (Fig. 3 and Supplementary_Table_2).

3.3.3. Genotypic characterisation of virulence

The entire panel of isolates showed high sequence similarity (more than 99% identity) with the virulence plasmid pSCV50, already described in the serovar Choleraesuis (Yu et al., 2006). In particular, all the *spv* operon genes and the toxin/antitoxin *ccdB/ccdA* cassette were present. These genes co-localised with IncFIB and IncFII replicons for 29 isolates (Supplementary_Table_2).

3.3.4. Identification of Salmonella Pathogenicity Islands (SPI)

A similar pattern of SPIs characterised the entire panel of genomes (Fig. 3 and Supplementary_Table_3). No differences were found between septicaemic and hunted animals isolates. Nevertheless, the wild boar isolate 12/90639 and the human isolate showed fewer SPIs than the other isolates.

3.3.5. Phylogenetic analysis

A total of 250 SNPs were identified among the panel isolates and used to construct a phylogenetic tree. Isolates clustered into two unrelated groups: one containing the two domestic pig isolates, and another gathering the septicaemic and hunted wild boar and the human isolates (Fig. 5). The two pig isolates presented 13 SNPs difference between them; on the contrary, the difference between the two groups exceeded 200 SNPs (Supplementary_Table_4).

Concerning wild boar and human isolates, a total of 69 SNPs were identified; nevertheless, the septicaemic and hunted wild boar isolates did not cluster into two distinct groups. Four genomes (the human isolate, the wild boar isolate containing the lowest number of SPIs and two wild boar isolates not carrying a recognisable somatic antigen sequence) presented more than 20 SNPs difference with the others. The other 27 wild boar genomes showed a very close relatedness, irrespective of their differences in expression of their entire antigenic formulae. In particular, the septicaemic wild boar isolates displayed an average 8 SNPs difference between themselves, while the hunted wild boar isolates showed an average number of 16 SNPs difference. The group of septicaemic genomes was characterised by a lower genetic variability than the hunted genome group (Supplementary_Table_4).

The phylogenetic analysis showed an average 44.7 SNPs difference between the human isolate genome and the investigated wild boar isolates (Supplementary_Table_4).

4. Discussion

The results of the present work display a scenario where the analysed epidemic case was caused by *S.* Choleraesuis var. Kunzendorf, which is considered the typical biovar of this serovar in swine infections (Pedersen et al., 2015). Even though clinical signs of infections were reported only in a fraction of the infected wild boar population (the septicaemic animals), PFGE analysis showed that overall, 76 wild boar isolates (from both dead septicaemic and asymptomatic hunted animals) were closely related (cluster I and II), thus probably differing by only a single genetic event (Tenover et al., 1995). Coherently, the SNP analysis grouped together all wild boar isolates, thus displaying a scenario where a unique *S.* Choleraesuis var. Kunzendorf might circulate within the wild boar population. The results of phylogenetic analysis showed that the presence/absence of the entire antigenic formula gene sequence did not infer any specific clustering to the panel of isolates.

The genotypic analysis of virulence determinants showed that isolates belonging to symptomatic and hunted animals displayed the same,

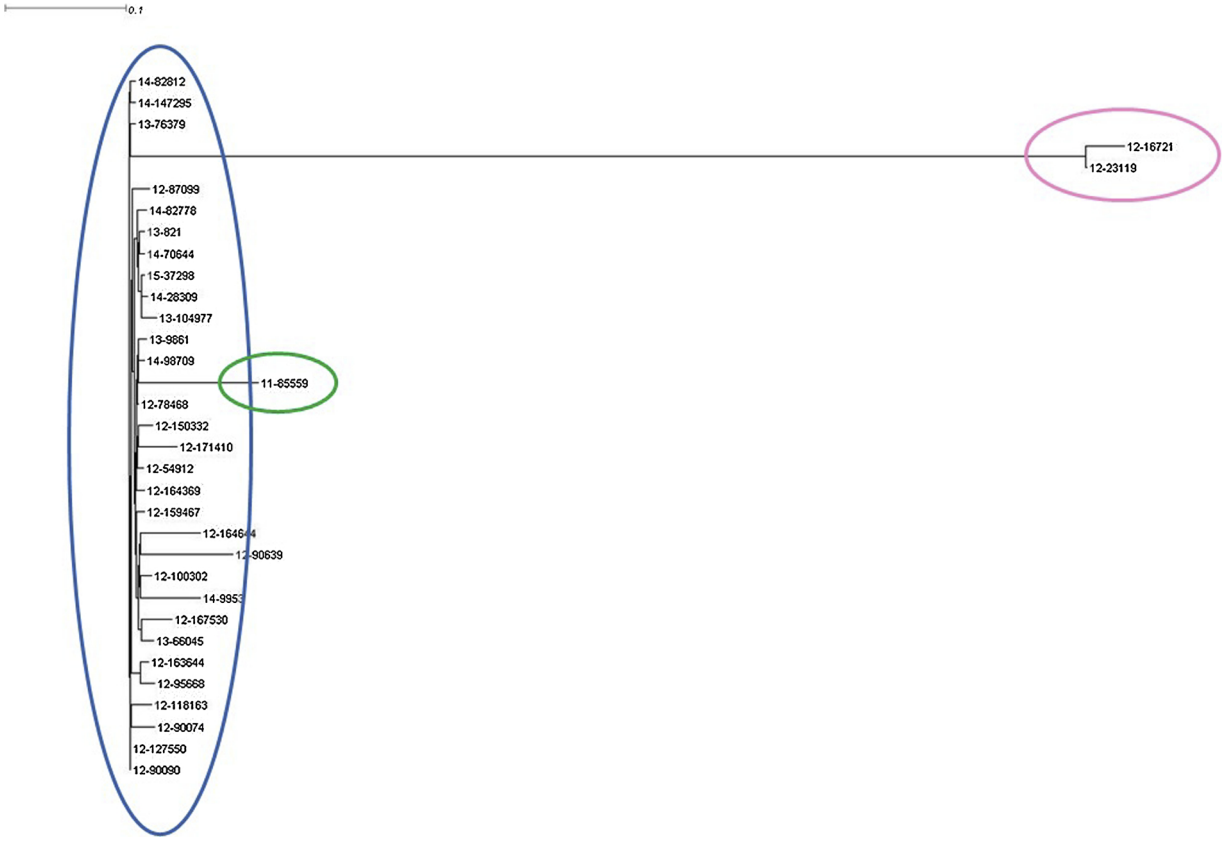

Fig. 5. SNP-based phylogenetic tree, built using CSIPhylogeny (version 1.4)[32] (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) and *S.* Choleraesuis strain SC-B67 (AE017220) as reference genome. The two domestic pig isolates are highlighted with pink colour, the septicaemic and hunted wild boar isolates with blue and the human isolate with green.

indistinct genomic virulence pattern. This supports the hypotheses of a role for the individual animal features in host-pathogen interaction and/or the role of co-infection with other agents in causing symptomatic salmonellosis.

All genomes studied contained a similar pattern of SPIs, with SPI-1 and SPI-2 being consistently present in all isolates. SPI-1 and SPI-2 are specific *Salmonella* determinants of virulence and contain the type three secretion system (T3SS). While SPI-1 is involved in the invasion of epithelial cells by *Salmonella*, SPI-2 is essential for *Salmonella* survival in macrophages (Lee et al., 2015). SPI-2 is also responsible for the translocation of SpvB and SpvC into the host cell cytoplasm (Guiney and Fierer, 2011).

The 50-kb virulence plasmid pSCV50, already characterised in *S.* Choleraesuis (strain SC-B67) (Yu et al., 2006), and its *spv* operon containing *spvR, spvA, spvB, spvC, spvD* genes, strongly associated with strains causing non-typhoid bacteraemia (Guiney and Fierer, 2011), were found in the whole panel of investigated genomes. All these virulence genes co-localised with the plasmid replicons IncFIB(S) and IncFII(S) in almost all the investigated genomes. The IncFIB(S) and IncFII(S) sequences are well known to be associated and co-localised on *Salmonella* virulence plasmids (Carattoli et al., 2014).

In addition, all the isolates harboured the toxin/antitoxin *ccdA/ccdB* cassette, located on the same contig containing both IncFIB(S) and IncFII(S) replicons and the *spv* operon that is known to be involved in plasmid stability through a mechanism of post-segregational killing (PSK) (Di Cesare et al., 2016; Harms et al., 2018).

The septicaemic and hunted wild boar isolates, besides having identical virulence markers, shared similar plasmid replicon types and were characterised by the absence of antimicrobial resistance genes, thus implying these isolates were not exposed to environmental selective pressures towards antimicrobials.

The isolate of *S.* Choleraesuis of human origin showed similar characteristics to the wild boar isolates, with particular reference to the absence of antibiotic resistance genes, and to the plasmid typing, virulence genetic determinants and pulsotype. However interpreting these findings in terms of a possible epidemiological relationship with the investigated outbreak is still challenging, as this human case preceded the outbreak in wild boar, and few anamnestic data of the patient were available. Nevertheless, the similarity between the human and wild boar isolates is reminiscent of the possible role of *S.* Choleraesuis as a human pathogen in Western countries, possibly due to human exposure *via* environment and/or food.

Concerning the possibility of infection spread from wild to domestic swine or *vice versa*, two septicaemic domestic pig isolates, from two swine farms located in a geographic region bordering the outbreak, were included in the analysis. These isolates shared the same biovar, MLST profile and the virulence genetic determinants with the wild boar and human isolates we investigated. However, the domestic pig isolates displayed a weak degree of epidemiologic correlation according to PFGE results, which clustered the pig isolates in a different group than the wild boars ones (Tenover et al., 1995; Barrett et al., 2006; Barco et al., 2003). In addition, based on genomic analyses, the pig isolates displayed a different plasmid replicon profile and were characterised by the presence of many antibiotic resistance genes, conferring resistance to aminoglycosides, beta-lactams, sulphonamides, tetracyclines and trimethoprim. Some of these genes (*aadA1, aph (6) -Id, aph (*3 '') *-Ib, sul2, sul1* and *dfrA1)* co-localised with the plasmid replicon IncQ1, suggesting a plasmidic localisation for such determinants. This result suggested that strains from domestic and wild pigs were never in touch, as mobile genetic elements, i.e. plasmids, which are spread in swine farm isolates (Panzenhagen et al., 2018), were not transferred to wild boar *Salmonella* isolates. Moreover, this result strongly points to the use of antimicrobials in pig farming probably being able to select for antimicrobial resistance genetic determinants in the residing microbial community, including pathogens. The phylogenetic analysis of SNPs showed a close relationship between the two domestic pig isolates, which formed a second cluster that differed from the SNP cluster with wild boar outbreak isolates and the human isolate, thus suggesting domestic pig isolates did not share any genetic correlation with these other isolates, as was also determined by our PFGE analysis.

Even though in the present study the differences between isolates were measurable without SNP analysis, as PFGE was discriminatory enough to present the true relationships among isolates, no information regarding the genetic context of the isolates, such as virulence genes, antimicrobial resistance determinants or plasmids, can be obtained by using this banding pattern characterisation method. Therefore, WGS-based analysis can certainly provide invaluable information regarding isolates' genetic characterisation that, in combination with epidemiological metadata, would assure accurate outbreak investigations.

Author contributions

Design and conception of the study: A.L., C.L., G.C., C.V.C., A.R., L.B. and A.A.L.; Isolate collection: S.T., D.V., G.C., C.V.C.; Methodology and data analysis: A.L., F.V., S.T., S.P., E.M., P.Z., M.C.D.P. and E.R.; Visualisation: C.M.; Writing of the manuscript: A.L., C.L., C.V.C, L.B. and A.A.L; All authors approved the manuscript.

Data availability statement

Raw sequence data were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession number PRJEB27935.

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Declaration of Competing Interest

The authors declare no competing interests. The authors wish to acknowledge Sheryl Avery (http://absees-editorial.my-free.website/contact-us) for linguistic revision of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108423.

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