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Phenotypic and genetic characterization of antimicrobial resistance in *Salmonella enterica* serovar Choleraesuis isolates from humans and animals in Spain from 2006 to 2021

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Objectives: While an increase in the levels of MDR in *Salmonella enterica* sevorar Choleraesuis has been reported in Europe, little is known about the situation in Spain. Therefore, we first aimed to assess the phenotypic resistance profile and to determine the presence of genetic determinants of resistance of *S.* Choleraesuis isolates collected in animal and human. Our second objective was to identify and characterize clusters of highly related isolates.

Methods: We analysed 50 human and 45 animal isolates retrieved from 2006 to 2021 using the disc diffusion method and performed WGS followed by analyses of genetic determinants and phylogenetic analysis.

Results: All isolates were of ST145 and corresponded to the variant Kunzendorf. Swine isolates harboured a significantly higher number of antimicrobial resistance genes than human isolates, and often carried plasmid replicons of the IncHI2/IncHI2A type (42% of all animal isolates). In addition, we identified several MDR *S.* Choleraesuis strains circulating in humans and swine between 2006 and 2021. The phylogenetic analyses identified four clades associated with specific patterns of resistance genes and plasmid replicons. The clades also included isolates that differed in terms of year and region of isolation as well as host of origin.

Conclusions: This One Health approach highlights that reducing human MDR *S.* Choleraesuis infections may require the adoption of strategies that not only seek to prevent cases in humans but also to characterize and reduce the infection burden in swine.

Introduction

Salmonella enterica subsp. *enterica* serovar Choleraesuis (*S.* Choleraesuis) is known to cause an often-fatal systemic disease, known as swine paratyphoid fever, in domestic pigs and wild boars.[1] *S.* Choleraesuis variant Kunzendorf is highly prevalent in North America and Asia but is relatively rare in the EU and Australia. However, multiple outbreaks in wild boars and pig herds have been recently reported in Europe including Serbia,[2] Denmark,[3] Sweden,[4] Italy[5] and Spain.[6] Wild boars were hypothesized to play a prominent role in the interplay between *Salmonella,* livestock and the human population.[7] Indeed, despite its relatively low prevalence in pigs, *S.* Choleraesuis is becoming more prevalent in wild boars from Europe, whose population has increased during the last decades.[8] However, results from molecular typing have failed to clearly establish a link between wild boar and domestic pigs.[5,9]

Although infections due to *S.* Choleraesuis in humans are unusual in Europe and most human cases are reported in Asia, they can be particularly severe and can be associated with septicaemic disease.[10] Recently, invasive disease was reported in 56.4% of human cases.[2] Because of its septicaemic character, antimicrobial therapy is necessary for the treatment of *S.* Choleraesuis infections. However, MDR *S.* Choleraesuis clones are increasingly being reported in Europe and worldwide. For example, a study from 2009 revealed that 87.5% of Irish swine isolates of

S. Choleraesuis were resistant to at least one antibiotic, whereas 37.5% showed resistance to four or more antibiotics.[11] This is especially concerning when it involves resistance to fluoroquinolones and third-generation cephalosporins, which are indicated for the treatment of invasive infections. A recent study in Thailand found that around 19% of *S.* Choleraesuis isolates from patients with systemic salmonellosis were resistant to at least one of nine cephalosporin antimicrobials and 15% were resistant to ciprofloxacin.[12] However, data on the antimicrobial profile of human isolates are still scarce in Europe and have been rarely investigated in Spain.

In the last decade, WGS has emerged as the method of choice for in-depth genomic characterization and typing of microbial pathogens, but also for the study of antimicrobial resistance (AMR). It allows the study of possible transmission clusters and/or to identify a common source among isolates. In 2018, WGS was performed to study outbreaks of *S.* Choleraesuis in wild boars from southwestern Spain and authors found that the isolates harboured a variety of AMR determinants.[6] However, there is still a need to characterize the genetic basis for AMR and to investigate the *S.* Choleraesuis strains circulating at the human-animal interface in Spain.

In this context, we aimed to identify the AMR profiles, AMR genes and genetic relatedness of *S.* Choleraesuis isolates retrieved from human cases, food and animal samples from 2006 to 2021 in Spain. To reach that goal, we coupled phenotypic antimicrobial susceptibility testing with whole genome sequencing followed by phylogenetic analyses.

Material and methods

Study population

The study population was formed by all ($n=50$) *S.* Choleraesuis human cases isolated received at the Spanish National Center of Microbiology (CNM) in Spain between 2008 and 2021. In addition, a total of 45 isolates of animal origin, including food isolates, available at the VISAVET Health Surveillance Center and at the CNM were included in the study. These isolates had been retrieved as part of diagnostic, surveillance and research activities at VISAVET-UCM and CNM between 2006 and 2021. More details can be found in the Supplementary Materials (Table S1, available as Supplementary data at *JAC* Online).

Antimicrobial susceptibility testing

Susceptibility to antimicrobials was determined with disc diffusion assays using Mueller–Hinton agar and commercially available discs (Oxoid™). Testing was carried out for a total of 18 antibiotics belonging to nine antimicrobial classes (Supplementary Materials, Table S2). Isolates were designated as resistant (R), susceptible, increased exposure (I) or susceptible (S) on the basis of available clinical breakpoint data published by the EUCAST (v.13.1). For antimicrobials in which such breakpoints were not available, we used CLSI Guidelines.[13] Multidrug resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial classes.

To confirm the phenotypical resistance to colistin of the six samples harbouring the *mcr-1.1.* gene (see next), the broth microdilution method was used. The minimum inhibitory concentration (MIC) was determined for 14 antimicrobial agents belonging to 10 antimicrobial classes (Supplementary Materials, Table S2) using Sensititre™ standard susceptibility MIC plate EUVSEC (TREK Diagnostic Systems/Thermo Fisher Scientific) according to the manufacturer's protocol.

Short read whole genome sequencing and in silico multi-locus sequence typing (MLST)

Genomic DNA from animal and human isolates was purified using the kit Maxwell® RSC Cultured Cells DNA Kit (Promega). Libraries were prepared by using Nextera XT DNA Library Preparation Kit and sequenced on a MiSeq platform by using version 3 reagents for 2×300 paired-end libraries (both from Illumina, https://www.illumina.com). Raw reads were uploaded on the EnteroBase online platform for *Salmonella* and genomes are available under the following Uberstrains names: SAL_OB9295AA to SAL_OB9715AA. Using the *Salmonella* scheme from EnteroBase, we performed an *in silico* MLST analysis based on seven housekeeping gene loci (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA*) to identify the sequence type.

Determinants of resistance and plasmid content

Quality of reads was checked using FastQC v.0.11.8[14] and assemblies were generated using Unicycler v.0.4.6[15]. Quality of the genome assemblies was check using Quast v.4.1[16] and Kmerfinder v.3.1[17,18] was used to identify the best matching reference. Then, assemblies were screened for the presence of antimicrobial resistance genes (ARGs) using the online ResFinder v.4.1. tool[19] with default settings from the Center for Genomic Epidemiology of the Technical University of Denmark (DTU). The identification of plasmid replicons was carried out using the online PlasmidFinder v.2.1. tool[20] with an identity threshold of 95% and a sequence coverage of 95% as a cut-off.

Raw reads of a selection of isolates were also screened for plasmid identification using PlasmidID (https://github.com/BU-ISCIII/plasmidID). Isolates for this analysis ($n=19$) were selected to be representative of the Bayesian Analysis of Population Structure (BAPS) clades (see next), the plasmid replicons according to PlasmidFinder, the host and year of isolation. All isolates carrying the *mcr-1.1.* genes were also included. For more details, see the Supplementary Materials.

Phylogenetic analysis

Core genome SNPs (cgSNPs) were called using Snippy v.4.4.5 (https://github.com/tseemann/snippy) and the genome of *S.* Cholerasuis strain CFSAN022628 (GenBank accession number CP075026.1) as a reference.[21] We considered a SNP matrix or more than 4 million nucleotides with around 2400 variant positions across all samples. We did not identify a significant number of SNPs in recombination regions when using Gubbins v.3.3.1 (https://github.com/nickjcroucher/gubbins). A ST68 *S.* Choleraesuis isolate (EnteroBase SAL_OB9633AA) was included as an outgroup in the analysis. The obtained alignments were analysed to build a maximum-likelihood phylogenetic tree using IQ-TREE v.2.1.4, in which the K3P(u)+F+I substitution model was used for SNP-only alignments, using the ultrafast bootstrap option (1000 replicates).[22,23] The three was visualized in iTOL.[24]

The presence of phylogenetical clades was evaluated by analysing the SNP alignment with RhierBAPs[25] in R v.3.6.1 (www.r-project.org). RhierBAPs is a BAPS software, which enables hierarchical clustering of DNA sequence data to reveal nested genetic population structures.

Statistical analysis

Pairwise Fisher's exact (pairwise_fisher_test; rstatix package) tests with Benjamini–Hochberg correction[26] and χ^2 tests (chisq.test; stats package) were used to compare frequencies of isolates resistant to all antibiotics depending on their origin (human versus swine) but also to compare BAPS clades in terms of frequencies of isolates from certain hosts, regions and year of isolation. Because of the low number of food and wild boar isolates, they were excluded from the statistical analyses. A Kruskal–Wallis test (kruskal_test; *rstatix* package) was applied to assess differences in the number of AMR genes of isolates depending on their BAPS clade. A *P* value cut-off of 0.05 was used to determine significance in all statistical analyses. Statistical analysis and visualization were performed in R v.3.6.1.

Results

Phenotypic antimicrobial profiles and antimicrobial resistance genes (ARGs)

Antimicrobial susceptibility testing (AST) was carried out on all the 95 isolates from human and animal origin. All *S.* Choleraesuis isolates were susceptible to cephamycin (third-generation cephalosporin) and there was only one isolate resistant to carbapenem. However, 77% of the isolates were resistant to at least one antimicrobial agent. Different determinants conferring resistance to aminoglycosides, beta-lactams, quinolones, tetracyclines, sulfamethoxazole, trimethoprim and colistin were found in the sequenced strains. Each isolate harboured between one and 13 ARGs (median = 4). Swine isolates harboured a significantly ($P = 0.0036$) higher number of ARGs than human isolates [median of 7 ARGs (IQR = (3.3; 11)] in swine versus 4 [IQR = (1; 7)] in human isolates). However, the proportion of MDR isolates among the different sources (46% and 66% of human and swine isolates, respectively) was not significantly different. Finally, the number of ARGs carried by the isolates or the number MDR isolates did not significantly increase over time. Phenotypic resistance to kanamycin, gentamicin and streptomycin were found in 2%, 16% and 45% of the isolates, respectively. However, we observed that 49% of the isolates required increased exposure (I) to streptomycin and therefore only two isolates were fully susceptible to all three of the aminoglycosides tested. Isolates retrieved from swine showed higher levels of resistance to gentamycin compared with those from humans ($P = 0.019$; Figure 1). A total of 10 determinants conferring resistance to aminoglycosides [*aac(6')-Iaa*, *aac(3)-IIa*, *aac(3)-IId*, *aac(3)-IV*, *aadA1*, *aadA2*, *aadA2b*, *aadA3*, *aph(3'')-Ib*, *aph(4)-Ia*, *aph(6)-Id*] were found in the isolate collection in addition to the cryptic gene *aac(6′)-Iaa*, not associated with phenotypic resistance. Up to six of these genes were present in 13 isolates. All but two phenotypically susceptible isolates had at least one aminoglycoside resistance determinant in addition to *aac(6′)-Iaa*.

Phenotypic resistance against folate pathway inhibitors was found in 59 (62%) isolates with 57 isolates resistant to sulfamethoxazole and 42 isolates resistant to trimethoprim. The percentages were not significantly different between swine and human isolates. Resistance gene *sul2* was found in 35 (37%) isolates (in combination with *sul1* in three isolates), whereas *sul1* and *sul3* were present in 16 and 9 isolates, respectively.

Twenty-two (23%) isolates carried the *dfrA1* gene, and 16 (17%) and three (3.2%) isolates harboured *dfrA14* and *dfrA12*, respectively, all of which were phenotypically resistant to trimethoprim.

Forty-eight (52%) isolates harboured the beta-lactamase-encoding gene $bla_{TEM\text{-}1B}$, and all were phenotypically resistant to ampicillin. Only one isolate (VE08/00559SM2) showed two additional beta-lactamase-encoding genes ($bla_{TEM\text{-}102}$ and $bla_{TEM\text{-}182}$). Human and swine isolates did not significantly differ in their resistance to ampicillin.

Twenty-seven (28%) isolates were phenotypically resistant to chloramphenicol and resistance was mediated by *cmlA1* in four isolates, by *catA1* in five isolates and by *floR* in 19 isolates.

Tetracycline phenotypic resistance was found in 50% of the *S.* Choleraesuis isolates. The determinant *tet(A)* was found in 35 (38%) isolates while 11 (12%) isolates had the *tet(B)* genes. In three phenotypically tetracycline-resistant human isolates, we did not observe genes of resistance. Human and swine isolates did not significantly differ in their resistance to chloramphenicol and tetracycline.

Regarding fluoroquinolone resistance, none of the isolates were resistant to ciprofloxacin but 15 (16%) were resistant to quinolones (nalidixic acid or pefloxacin). The percentages were similar in human and swine isolates. All the resistant isolates presented a non-synonymous mutation in *parC* (codon T57S) conferring resistance to quinolones. In addition, the sequenced strains presented one of four mutations in codons 83 or 87 of the *gyrA* gene: D87N was found in nine isolates (9.75%; five human and four swine isolates), D87G in three isolates (3.2%; two human and one swine isolates), S83Y in five isolates (5.4%; four swine and one human isolates) and S83F in one human isolate. Two plasmid-mediated quinolone resistance (*PMQR*) genes were also found, *qnrB19* and *qnrS1* in VE08/00559SM2 (Iberian swine) and 20154287 (human), respectively.

Finally, the *mcr-1.1* gene was found in six isolates recovered from human ($n = 1$) and swine ($n = 5$), but only five isolates were phenotypically resistant (MIC >= 16 mg/L) to colistin using the Sensititre method. Those five isolates were also phenotypically resistant to azithromycin without carrying specific ARGs.

Plasmid replicons and identification of plasmids

The IncFII replicon was present in all isolates, and IncFIB was also present in 82 of them (86%). According to the PlasmidID analysis performed in 19 of them, these two replicons were carried by a plasmid almost identical (mapping percentage > 99%) to the *S.* Choleraesuis specific plasmid (pSCV50 identified in str. SCSA50) (Table 1; Figure 2a). All the 19 isolates analysed with PlasmidID were also found to harbour a plasmid similar to one identified in an isolate of the Senftenberg serovar but carried no plasmid replicon or resistance genes. The replicon IncHI2/IncHI2A was more prevalent in isolates of swine origin, with 42% of them

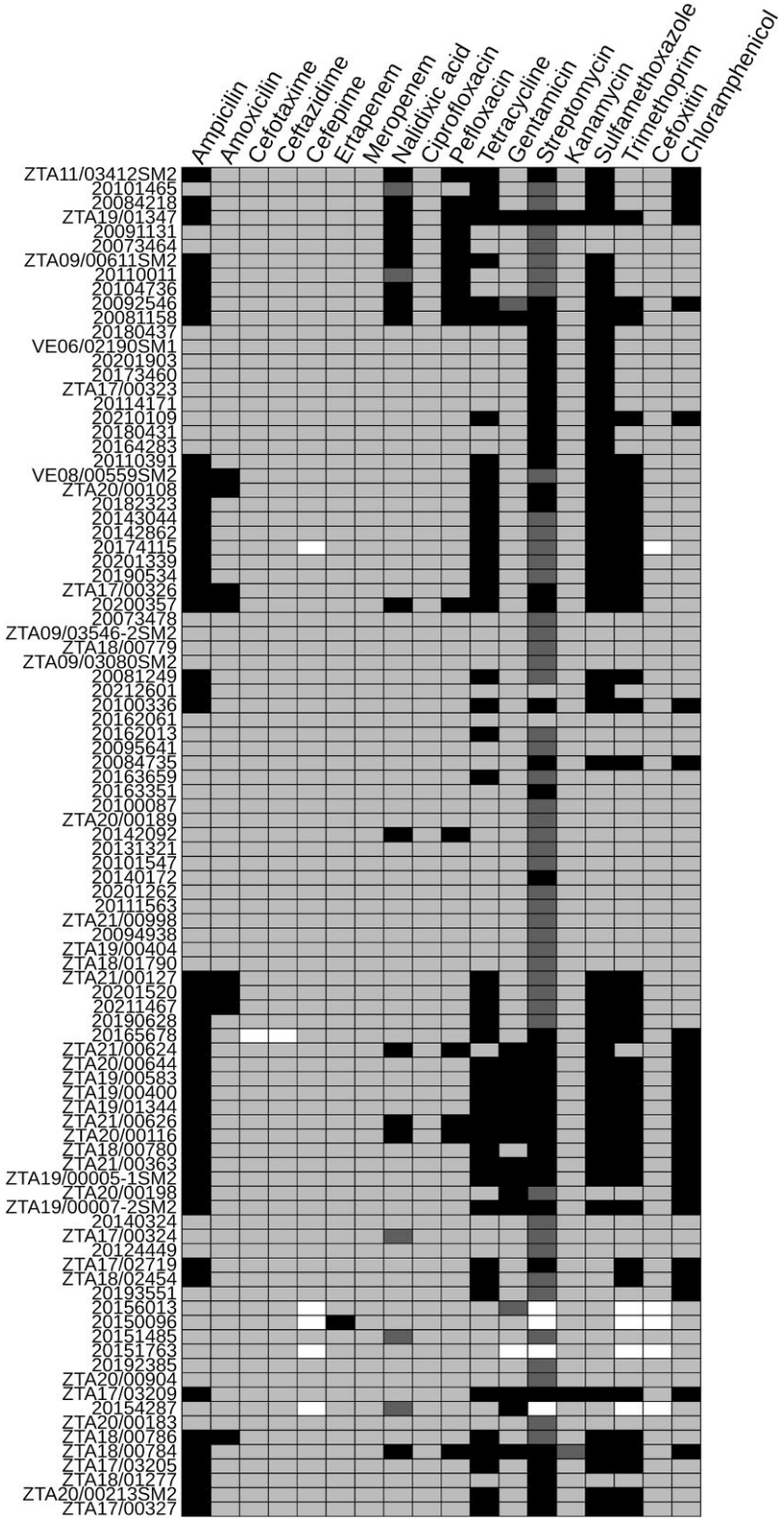

Figure 1. Heatmap of antimicrobial susceptibility of all the isolates included in the study. Black is used for resistance, dark grey for intermediate and light grey for susceptibility. Missing data are represented in white. Susceptibility to antimicrobial agents was determined using the Kirby–Bauer disc diffusion method and interpreted according to the clinical breakpoints of the EUCAST. Isolates are presented in the order of Figure 3.

Table 1. Characteristic of the plasmid of reference in the 19 isolates selected for PlasmidID analysis according to the plasmid replicons harboured by the isolates

Replicons	Number of isolates	Plasmid reference	Gene of resistance	Average mapping (%) [range]
IncFII, IncFIB	19 (8 human, 11 animal)	*S. enterica* Choleraesuis str. SCSA50 (pSCV50)		99.8 [97.6–100]
IncHI2, IncHI2A	3 (swine)	*E. coli* O157 str. 180-PT54	*aph(6)-Id*, *aph(3")-Ib*, *dfrA1*, *aadA1*, *sul1*, *tet(A)*	94.2 [93.6–94.6]
	3 (animal)	*S. enterica* str. CFSA629	*mcr-1.1*, *aph(6)-Ib*, *aac(3)-IV*	96 [95.3–96.6]
	1 (human)	*S. enterica* Heidelberg str. N13-01290	*sul1*, *aadA2*, *dfrA12*, *aph(6)-Ib*, *tet(B)*	87.7
	1 (human)	*S. enterica* Typhimurium str. 2470	*sul2*, *aph(3")-Ib*, *dfrA1*, *tet(A)*, *aadA1*	95
	1 (human)	*E. coli* str. CFSAN061770 (pEGY1-MCR-1)	*mcr-1.1*, *tet(A)*	89.7
IncN	3 (human)	*S. enterica* str. CFSAN064033 (pGMI17-001)	*dfrA14*, *aph(6)-Id*, *tet(A)*, *blaTEM-1B*	98.7 [96.3–99.9]
IncHIB, IncFIA	2 (1 human, 1 swine)	*E. coli* O111 str. 11128	*aph(3")-Ib*, *aph(6)-Id*, *sul2*, *tet(B)*	90.2 [90.1–90.3]
IncI1(Alpha)	1 (human)	*S. enterica* Typhimurium (pSal8934a)		98.2
	2 (swine)	*E. coli* O104:H4 str. C227-11		98.4 [98.4–98.4]
ColBS512	1 (human)	*S. sonnei* 53G (plasmid C)		100

harbouring both replicons compared to 10% of the human isolates. Among the 11 isolates with those replicons analysed with PlasmidID, we found that IncHI2/IncHI2A was carried by plasmid sequences similar to those previously encountered in different strains of *S. enterica* and *Escherichia coli* (Table 1). Plasmid sequences containing *mcr-1.1* genes were similar to sequences from *S. enterica* str. CFSA629 (Figure 2b), *E. coli* O157 and *E. coli* str. CFSAN061770 (Figure 2c). By contrast, replicon IncN was present in 18% of human isolates and 14% of animal isolates. Out of 15 isolates carrying the IncN plasmids, three were analysed with PlasmidID and all harboured a plasmid similar to the pGMI17-001 plasmid (*S. enterica* strain CFSAN064033).

Epidemiological and microbiological profile of phylogenetical clades

One human isolate was excluded from the analysis due to low sequence quality. *In silico* serotyping and MLST confirmed the identity of the remaining 94 isolates as *S.* Choleraesuis ST145 belonging to the variant Kunzendorf.

According to the phylogenetic analysis based on the SNP multi-alignment (2427 bp length), four BAPS clades containing between nine (clade 1) and 63 (Clade 4) swine, food, wild boar and human isolates were identified (Figure 3). Isolates from Iberian swine were not observed in Clade 2, those from wild boar and eggs were only in Clade 4, and the ones from non-Iberian swine were present in all four clades (Table 2). Out of 25 isolates from 2006 to 2011, 32% belonged to in Clade 1, which was a significantly higher proportion than expected especially considering that the Clade 1 represented only 9.6% of all isolates (χ^2; $P=0.0018$). Among the isolates collected from farms in the west of Spain, 33% were associated with Clade 3 which was significantly higher than expected (χ^2; $P=0.0004$). On the other hand, 81% of the isolates coming from provinces in the south-east region belonged to Clade 4. Finally, isolates from Clade 3 had a significantly higher number of ARGs than other clades ($P=0.009$), and it also showed a higher proportion of MDR isolates compared to other clades, as all the isolates from this clade were MDR ($P=0.003$).

For genes determining resistance to aminoglycosides, all the isolates from Clade 4 carried the gene *aac(3)-IV*, which was absent from the other clades. In addition, only one swine isolate out of Clade 4 harboured the gene *aph(4)-Ia.* By contrast, 82% to 90% of isolates carried the genes *aph(3")-Ib* and *aph(6)-Id* in Clade 2 and 3, respectively. In addition, all the isolates from Clade 3 carried the genes *blaTEM-1B* and *tet(A),* while *tet(A)* was absent was isolates in Clade 1 and 2. In Clade 1, we found that 54% of the isolates carried the *tet(B)* gene, which was absent from Clade 3. Out of 23 isolates harbouring the gene *dfrA1*, only two were found outside of Clade 4 whereas 90% of the isolates from Clade 3 carried *dfrA14*. All the isolates from Clade 1 showed the same *gyrA* (D87N) mutation that was absent from the other clades. The mutation D87G was present in one isolate from Clade 2 and two isolates from Clade 4. While the mutation S83F was only present in one isolate of Clade 3, S83Y was present in four isolates of Clade 4 and one isolate of Clade 2. All the isolates presenting *mcr-1.1*. were in Clade 4. The gene *sul2* was harboured by all the isolates of Clade 2 and 3 whereas seven (78%) isolates from Clade 1 carried *sul3*. Finally, only one isolate out of the 16 isolates carrying *sul1* was outside of Clade 4.

All the isolates of Clade 3 harboured the plasmid replicon IncN, which corresponds to 73% of all the isolates with the replicon. Clade 4 contained all the isolates with IncI1-I. The IncHI2/IncHI2A replicon was present in 35% of the isolates from Clade 4 but in only one isolate outside of this clade. Out of the seven isolates with the IncFIA replicon, three were present in Clade 1.

Discussion

WGS and AST were used to analyse *S.* Choleraesuis isolates retrieved from human, swine, wild boar and food samples in Spain from 2006 to 2021. Phenotypic AST revealed different

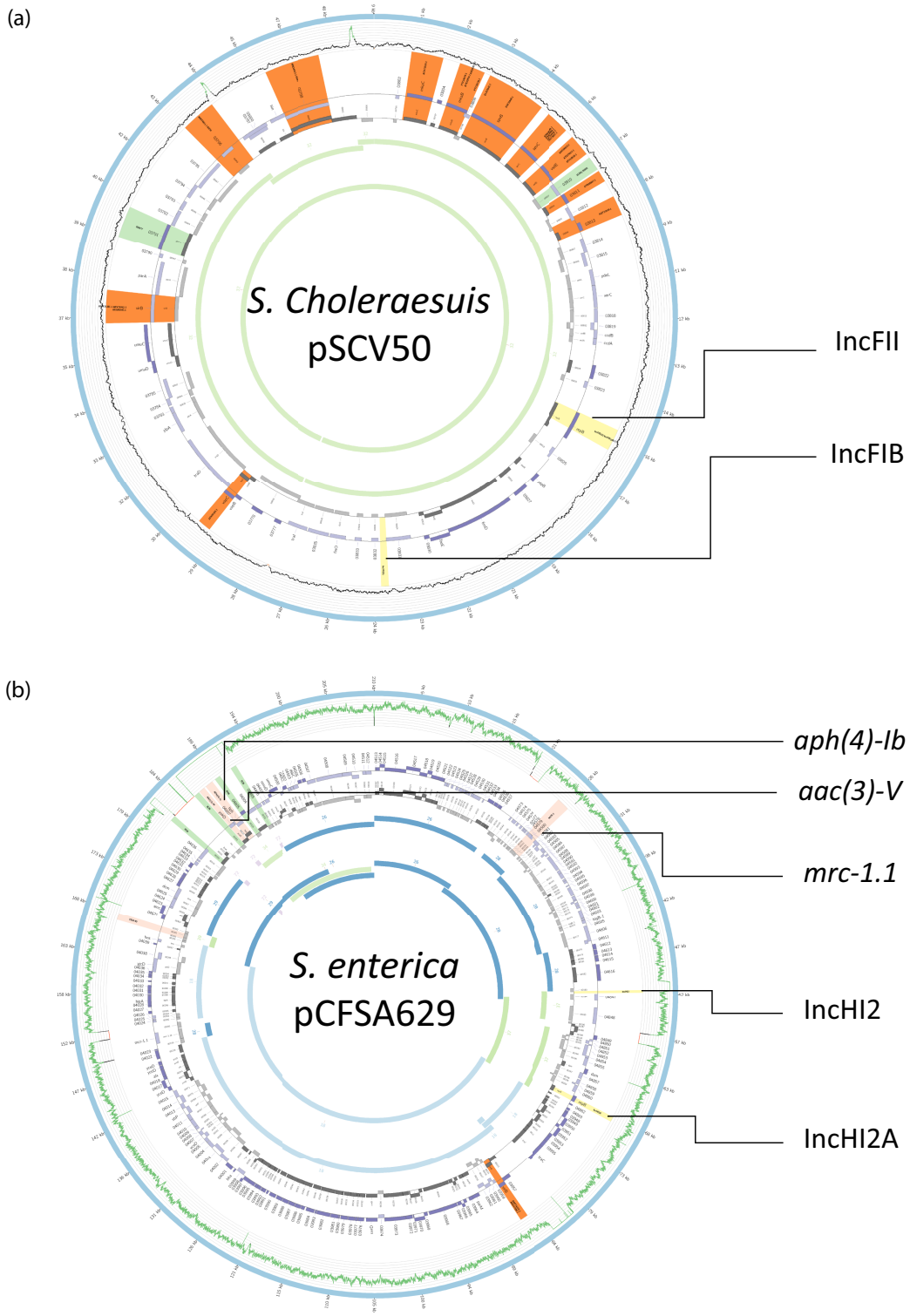

Figure 2. Mapping of isolates with (a) Choleraesuis specific plasmid (pSCV50), (b) *S. enterica* subsp *enterica* plasmid (pCFSA629) and (c) *E. coli* plasmid (pEGY1) performed by PlasmidID. Annotations for resistance genes and plasmid replicons are indicated. From the outside track to the inner track: (i) reference plasmid size indicators, (ii) histogram with the number of reads mapped in each position of the reference plasmid, (iii) two layers including contig and plasmid database annotation results from Prokka software. Each purple and grey box indicated a predicted named gene or protein coding sequence with its identity tag placed over the sequence, for contigs and plasmid database respectively. Genes over the line and darker are located in + strand, the rest in—strand, (iv) the contig track represents local alignments of contigs sequences that match plasmid region and (v) the complete contig track represent the full length of contigs that are homologous to the reference plasmid.

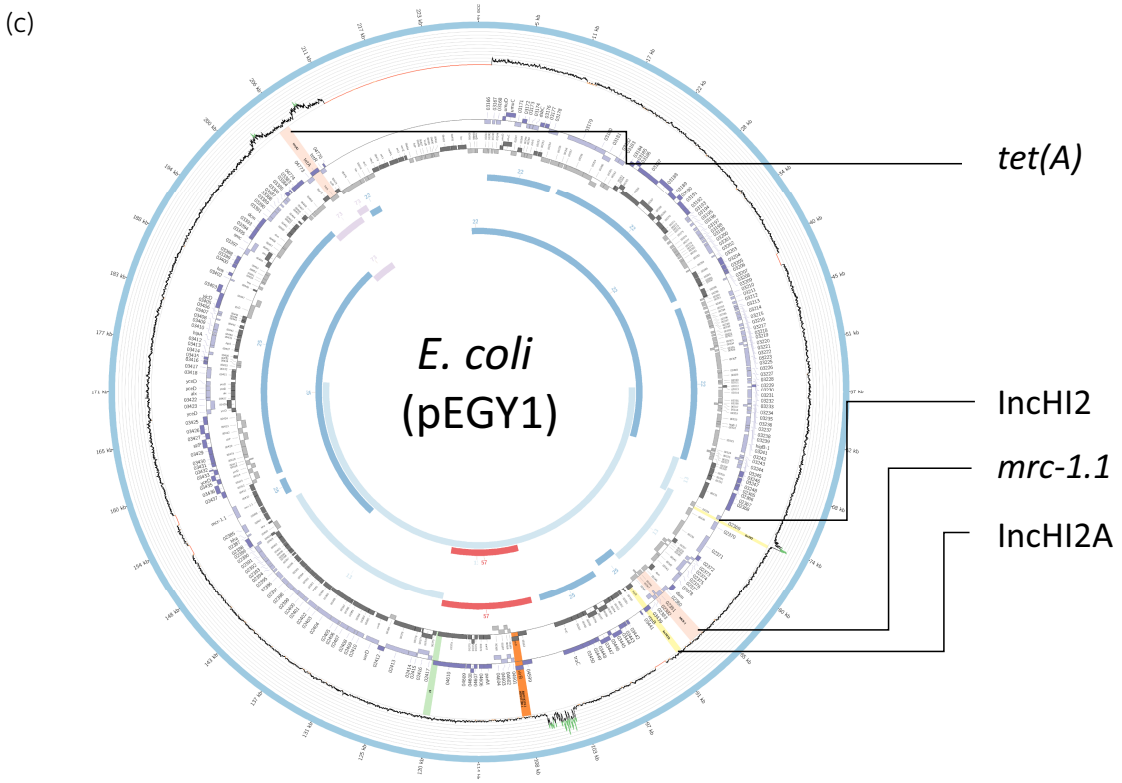

Figure 2. Continued

profiles of resistance in human isolates and isolates of animal origin. Swine isolates presented high resistance levels (>60% of isolates), especially to ampicillin, trimethoprim-sulfamethoxazole and aminoglycosides, and were associated with a higher number of ARGs than human isolates. This could be related to the past use of sulphonamides alone or in combination with tetracyclines in the prophylaxis and treatment of diverse pathologies that can affect pig farms. A similar result was obtained in Danish pig herds[3] and in swine isolates from the USA, where resistance to tetracyclines reached extremely high levels (92.6%).[27] However, another hypothesis could rely on the selection of AMR following the use of antibiotics in the sampled animals, since most of them were retrieved from clinically affected animals. However, information on whether treatment was applied (and if so, what antibiotics) was not available.

In humans, fluoroquinolones, azithromycin and third-generation cephalosporins are the recommended treatment for invasive *Salmonella*. Interestingly, we did not find resistance to third-generation cephalosporines or fluoroquinolones. Colistin is authorized for its use in veterinary medicine but consumption in pig farms has decreased by 97% between 2016 and 2019 to counter the increase in resistance observed in pigs.[28,29] Even though usage in humans is limited, colistin has been re-introduced as a last-resort treatment option in the context of the recent worldwide MDR increase. We identified a small cluster including human and animal isolates from 2016 to 2020 harbouring the *mcr-1.1* gene on an IncHI2-type plasmid. Our results confirmed a previous study that found an Iberian isolate from 2020 with a plasmid carrying the *mcr-1.1* gene.[30] The circulation of such plasmids is of concern for the clinical management of *S.* Choleraesuis and other enterobacterial species in both humans and animals. Indeed, it has already been suggested that the *mcr-1.1* gene can be transferred and carried by other plasmids than IncHI2, such as IncI2-type, IncX4 and IncP[31,32] and in other species, such as *E. coli*. Therefore, isolation and early treatment of *mcr-1.1* cases should be implemented to avoid the spillover of this plasmid to others enterobacterial species.

Some isolates from serovar Choleraesuis are known to harbour a virulence plasmid (pSCV50) of 50 kb in size,[33] which carries the replicons IncFII and IncFIB.[34] pSVC50 was highly similar to the plasmid sequences found in all the isolates tested with PlasmidID. It was described in the literature that pSCV50 can carry antibiotic-resistance genes, notably *sul1* and *blaTEM-1* coding for resistance to sulfonamide and ampicillin, respectively.[33] However, in our study the pSCV50-like sequences did not harbour these resistance genes. Rather, we found that resistance determinants were linked to other plasmid replicons (IncHI2/IncHI2A, IncHI1B, IncF1A and IncN). These replicons were previously found in isolates from other serovars (Heidelberg, Typhimurium) or from *E. coli*.[35] Especially, IncHI2 plasmids was involved in the acquisition and dissemination of ARGs in *Salmonella* and other Enterobacteriaceae.[36] Therefore, the detection of IncHI2/IncHI2A replicons together with several ARGs in putative plasmid sequence in 42% of isolates of animal origin is a worrying finding since the transmission of those plasmids could lead to decreased antimicrobial susceptibility.

Figure 3. Maximum-likelihood phylogenetic tree of 94 *S. Choleraesuis* ST145 isolates. Sequences were aligned to genome CP075026. The coloured labels indicate the four identified clades [groups identified with BAPS (level 1)]. The following information is presented to the left of the isolate IDs: host of origin, region of collection, presence/absence of ARGs and plasmid replicons. Branches with a bootstrap values >90 support are shown in red in the tree. Isolates in red were selected for the PlasmidID analyses. Details on the possible events of transmission and the identification of prophages can be found in the Supplementary Materials.

Table 2. Number of isolates allocated to each of the clades identified in the phylogenetic analysis depending on host, region and year of collection, as well as median number of ARGs

Variables	Clade 1 $n=9$ (9.6%)	Clade 2 $n=11$ (12%)	Clade 3 $n=11$ (12%)	Clade 4 $n=63$ (67%)	Total $n=94$ (100%)
Host					
Animal	4 (9.3%)	2 (4.6%)	5 (12%)	32 (74%)	43
White swine	2 (28%)	2 (28%)	1 (14%)	2 (28%)	7
Iberian swine	1 (3.8%)	0	3 (11%)	22 (85%)	26
Wild boar	0	0	0	2 (100%)	2
Unknown	1 (12%)	0	1 (12%)	6 (75%)	8
Human	5 (10%)	9 (18%)	6 (12%)	29 (59%)	49
Female	3 (16%)	3 (16%)	2 (10%)	11 (58%)	19
Male	2 (6.6%)	6 (20%)	4 (13%)	18 (60%)	30
Food (egg)	0	0	0	2 (100%)	2
Year range					
2006–2011	8 (32%)	4 (16%)	2 (8%)	11 (44%)	25
2012–2017	0	3 (11%)	4 (15%)	20 (74%)	27
2018–2021	1 (2.4%)	4 (9.5%)	5 (12%)	32 (76%)	42
Region					
North	4 (22%)	4 (22%)	1 (5.5%)	9 (50%)	18
South-east	1 (1.7%)	6 (10%)	4 (6.9%)	47 (81%)	58
West	3 (17%)	1 (5.5%)	6 (33%)	7 (39%)	18
ARGs					
Median (range)	5 (2–13)	4 (4–9)	7 (5–10)	3 (1–13)	4 (1–13)

Percentages are calculated according to the total number of isolates in the collection for each category.
North: Vizcaya, Guipúzcoa, Álava, Navarra, Leon, Huesca, La Rioja, Zamora, Valladolid. West: Tarragona, Castellón, Alicante, Murcia. East/South: Salamanca, Ávila, Madrid, Cáceres, Badajoz, Ciudad Real, Córdoba, Huelva, Sevilla, Granada, Málaga.
NB: Regions were defined in terms of density of swine and wild boars per municipality, with the south-east region showing the higher density of extensive pig farms [Registro de explotaciones ganaderas (REGA)]. The different periods of collection were based on global consumption of antibiotics in humans, where a decrease was observed since 2018 in Spain (PRAN, www.resistenciaantibioticos.es).

Phylogenetic analysis showed that human and animal isolates clustered together in each of the four BAPS clades, Clade 4 being associated with most of the sequences circulating in Spain. The clades showed different characteristics in terms of time, location of the isolate and host of origin but also regarding the number of ARGs and the plasmid replicons. A few outbreaks of *S.* Choleraesuis were previously reported in wild boars in the centre of Spain[6] and it was hypothesized that wild boars could act as reservoirs for *Salmonella*, acting as carriers and intermittent shedders.[37] Our results indicate that in Spain, Iberian and non-Iberian pigs could also be a reservoir of *S.* Choleraesuis and play a role on the human infections caused by this serotype, and thus should be considered in monitoring programmes along with wild boar.

In conclusion, the detection of AMR traits in *S.* Choleraesuis isolates from non-Iberian and Iberian swine highlights the importance of the interface between livestock, wildlife and anthropogenic environments. While Choleraesuis infections remain rare in Spain, the percentage of invasive disease and the need for antimicrobial treatment is high. Therefore, it is critical to strengthen microbiological surveillance and the One Health perspective to contain the spread of MDR strains in Spain and to other European countries. Our study also reinforces the idea that reducing MDR Choleraesuis infections in humans will require interventions in the food industry such as increasing control measures on hygiene and biosafety in pig farms and during transportation. Finally, by focusing on a serovar with a high impact on public health, our results also could permit the adaptation of clinical guidelines for antimicrobial treatment in patients infected with *S.* Choleraesuis, by prioritizing the use of ciprofloxacin over other quinolones as first line of treatment.

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Transparency declarations

Nothing to declare.

Supplementary data

Tables S1 to S3 are available as Supplementary data at *JAC* Online.

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