







## MDR *Shigella sonnei* in Spain: an ever-evolving emerging threat?

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**Background:** Seven CTX-M-27-producing *Shigella sonnei* strains were isolated at the University Hospital Virgen del Rocío (Seville, Spain) microbiology service from October to November 2021.

**Objectives:** To offer extensive information on the microbiological and molecular epidemiology results of the seven *S. sonnei* isolates and compare them with other previously documented CTX-M-27-producing *S. sonnei* associated with MSM transmission.

**Methods:** *S. sonnei* isolated from stool samples of patients with acute diarrhoea were identified through biochemical and serological typing. Whole characterization of the seven isolates was performed by sequencing with MinION Mk1C followed by genomic and molecular analysis.

**Results:** All the isolates were resistant to penicillins, cephalosporins, fluoroquinolones, cotrimoxazole and azithromycin. Sequencing showed the presence of several resistance determinants, outstanding *bla*<sub>CTX-M-27</sub>, azithromycin resistance genes [*ermB* and *mph(A)*], *qnrB19* and mutations in the QRDRs. All isolates belonged to the same hierarchical clustering of cgMLST (HierCC) with five allele distance (HC5) scheme v1 from Enterobase. However, they presented differences in plasmid composition, with all seven isolates harbouring IncFII, IncB/O/K/Z and ColE1-like while SH2, SH6 and SH7 had IncFIB only. Our isolates were closely related to others from Spain (HC5; 98748), Australia (HC5; 98748) and the UK (HC5; 98748), which were also associated with MSM transmission. Nevertheless, the structure of the non-chromosomal genetic elements and the genetic context of *bla*<sub>CTX-M-27</sub> presented a certain variability compared with isolates from other countries and among them.

**Conclusions:** This study confirms the emergence of CTX-M-27-producing *S. sonnei* (ST152) associated with MSM transmission in Spain, adding it to the Europe outbreak list and reinforcing the necessity of active surveillance and control of this high-risk clone.

### Introduction

Recently, the ECDC warned about the increase in extensively drug resistant *Shigella sonnei* infections in MSM in Europe and the UK.<sup>1</sup> From October to December 2021, seven cases of shigellosis caused by MDR *S. sonnei* strains were reported to the epidemiological surveillance system by the Microbiology Service of the University Hospital Virgen del Rocío (Seville, Spain). Therefore, the aim of this study is to provide detailed information of the microbiological results and molecular epidemiology of these strains and compare

them with others described, since there is an important increase in reported cases with high impact in public health at the international level.

### Material and methods

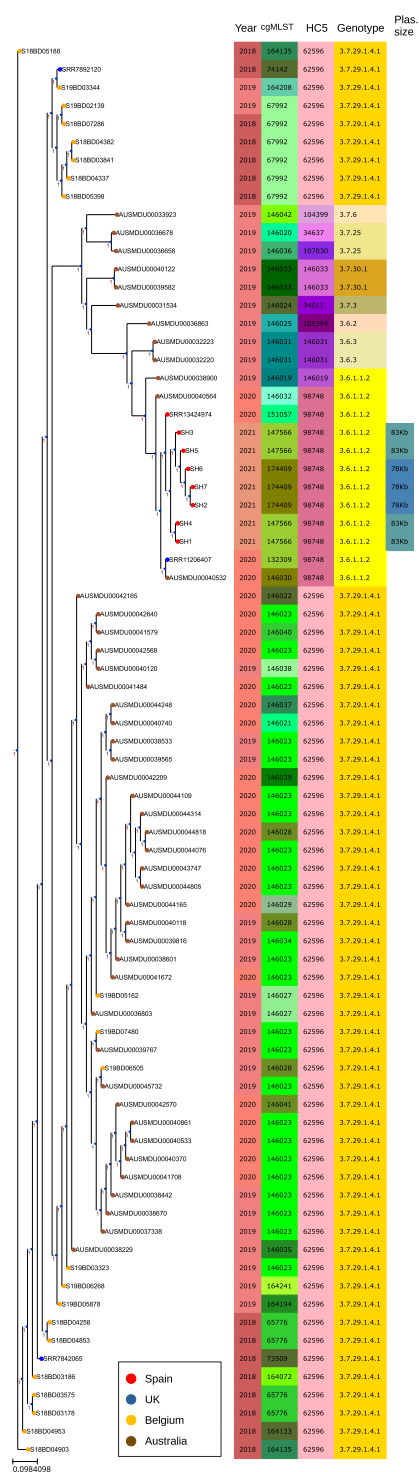
#### Patients

All patients presenting at the hospital or health centre with acute febrile gastroenteritis of unknown origin are asked to provide stool samples that

**Table 1.** Demographical, epidemiological, and microbiological data of patients and *S. sonnei* isolates

Demographic data											
Name	isolate date (day/month/ year)	gender	age (years)	cgMLST	Lineage	Clade	Genotype	Resistance phenotype	Resistance genes	Mutations	Plasmid
SH1	5/10/21	Male	35	147566	L3	3.6	3.6.1.1.2	AMP, CXM, CTX, CAZ, FEP, CIP, LVX, SXT, AZM	<b>bla<sub>CTX-M-27</sub></b> , <i>aadA5</i> , <i>aph(3'')-lb</i> , <i>aph(6)-ld</i> , <i>sul1</i> , <i>sul2</i> , <i>dfrA1</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>mdf(A)</i> , <i>ermB</i> , <i>tet(A)</i> , <i>qnrB19</i>	<i>parC</i> (S801), <i>gyrA</i> (S83L, D87G)	ColE1, <b>IncFII</b> , IncB/O/K/Z
SH2	4/11/21	Male	35	174409	L3	3.6	3.6.1.1.2	AMP, CXM, CTX, CAZ, FEP, CIP, LVX, SXT, AZM	<b>bla<sub>CTX-M-27</sub></b> , <i>aadA5</i> , <i>sul1</i> , <i>dfrA1</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>mdf(A)</i> , <i>qnrB19</i>	<i>parC</i> (S801), <i>gyrA</i> (S83L, D87G)	ColE1, <b>IncFII</b> , IncFIB, IncB/ O/K/Z
SH3	3/11/21	Male	30	147566	L3	3.6	3.6.1.1.2	AMP, CXM, CTX, CAZ, FEP, CIP, LVX, SXT, AZM	<b>bla<sub>CTX-M-27</sub></b> , <i>aadA5</i> , <i>aph(3'')-lb</i> , <i>aph(6)-ld</i> , <i>sul1</i> , <i>sul2</i> , <i>dfrA1</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>mdf(A)</i> , <i>ermB</i> , <i>tet(A)</i> , <i>qnrB19</i>	<i>parC</i> (S801), <i>gyrA</i> (S83L, D87G)	ColE1, <b>IncFII</b> , IncB/O/K/Z
SH4	7/10/21	Male	38	147566	L3	3.6	3.6.1.1.2	AMP, CXM, CTX, CAZ, FEP, CIP, LVX, SXT, AZM	<b>bla<sub>CTX-M-27</sub></b> , <i>aadA5</i> , <i>sul1</i> , <i>dfrA1</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>mdf(A)</i> , <i>ermB</i> , <i>qnrB19</i>	<i>parC</i> (S801), <i>gyrA</i> (S83L, D87G)	ColE1, <b>IncFII</b> , IncB/O/K/Z
SH5	7/11/21	Male	46	147566	L3	3.6	3.6.1.1.2	AMP, CXM, CTX, CAZ, FEP, CIP, LVX, SXT, AZM	<b>bla<sub>CTX-M-27</sub></b> , <i>aadA5</i> , <i>sul1</i> , <i>dfrA1</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>mdf(A)</i> , <i>ermB</i> , <i>qnrB19</i>	<i>parC</i> (S801), <i>gyrA</i> (S83L, D87G)	ColE1, <b>IncFII</b> , IncB/O/K/Z
SH6	16/12/21	Male	37	174409	L3	3.6	3.6.1.1.2	AMP, CXM, CTX, CAZ, FEP, CIP, LVX, SXT, AZM	<b>bla<sub>CTX-M-27</sub></b> , <i>aadA5</i> , <i>sul1</i> , <i>dfrA1</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>mdf(A)</i> , <i>qnrB19</i>	<i>parC</i> (S801), <i>gyrA</i> (S83L, D87G)	ColE1, <b>IncFII</b> , IncFIB, IncB/ O/K/Z
SH7	21/12/21	Male	36	174409	L3	3.6	3.6.1.1.2	AMP, CXM, CTX, CAZ, FEP, CIP, LVX, SXT, AZM	<b>bla<sub>CTX-M-27</sub></b> , <i>aadA5</i> , <i>sul1</i> , <i>dfrA1</i> , <i>dfrA17</i> , <i>mph(A)</i> , <i>mdf(A)</i> , <i>qnrB19</i>	<i>parC</i> (S801), <i>gyrA</i> (S83L, D87G)	ColE1, <b>IncFII</b> , IncFIB, IncB/ O/K/Z

**bla<sub>CTX-M-27</sub>**: CTX-M-27 encoding gene. **IncFII**: Plasmid IncFII that contains the *bla<sub>CTX-M-27</sub>* gene. AMP, ampicillin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole; AZM, azithromycin.



**Figure 1.** Phylogenetic tree with Spanish, European and Australian clones. Red text below the branch is the support. Red dots, Spanish isolates; blue dots, UK isolates; yellow dots, Belgian isolates, brown dots, Australian isolates. First column: isolation year. Second column: cgMLST based on EnteroBase *Escherichia/Shigella* cgMLST scheme. Third column: hierarchical clustering of cgMLST (HierCC) with five allele distance (HC5) scheme v1 from EnteroBase *Escherichia/Shigella* database. Fourth column: genotype, according to Hawkey *et al.*<sup>15</sup> Fifth column: CTX-M-27 plasmid size.

are processed according to epidemiological surveillance protocols in Spain. Shigellosis is a nationally notifiable condition to the epidemiological system subject to specific monitoring. Standard questionnaires were used to collect data on personal, clinical and microbiological characteristics and risk factors. If association of cases or a common origin is suspected, it is reported as a cluster or outbreak and considered a health alert. This cluster was reported after the identification of seven cases with similar microbiological and epidemiological characteristics, prompting a special investigation and a thorough review of microbiological *Shigella* isolates.

## Microbiological analysis

*S. sonnei* isolates were isolated from stool samples of seven patients from whom clinical data were included in the study after prior anonymization. *S. sonnei* isolates were identified by biochemical typing and serotyping. The susceptibility profiles were determined using MicroScan-Walkaway (Beckman Coulter, USA). ESBL production was determined by phenotypic testing following EUCAST recommendations.<sup>2</sup>

## Molecular analysis

Sequencing of the isolates was performed using a MinION Mk1C (Oxford Nanopore Technologies, UK) with a Flow Cell FLO-MIN106, extraction kit SQK-LSK109 and barcode kit EXP-NBD104. Basecalling and barcode trimming was performed in a GPU cluster with four Tesla-v100 using guppy-5.0.16 in high accuracy mode. In order to perform a genomic analysis, nanopore long reads were filtered with fastp-0.23.2<sup>3</sup> and assembled using flye-2.9<sup>4</sup> (Table S1, available as [Supplementary data](#) at JAC-AMR Online).

The clonal relationships between the seven *S. sonnei* isolated in this study and with the other available European and UK sequences were analysed from their assemblies using parsnp-1.2<sup>5</sup> to perform the alignment, core genome, SNP selection and phylogenetic tree. Then, Ete3 3.1.2<sup>6</sup> was used to draw the tree from newick file. Finally, for each sample, the cgMLST was calculated based on EnteroBase *Escherichia/Shigella* cgMLST scheme.<sup>7</sup> Circularized plasmids were obtained after the assembly process by flye-2.9. Then, CGE PlasmidFinder-2.0 and ResFinder-3.2<sup>8,9</sup> were used to detect the resistance determinants and identify the plasmid type that carried them from every single *Shigella* assembled sequence. The plasmids were also annotated by RAST.<sup>10</sup>

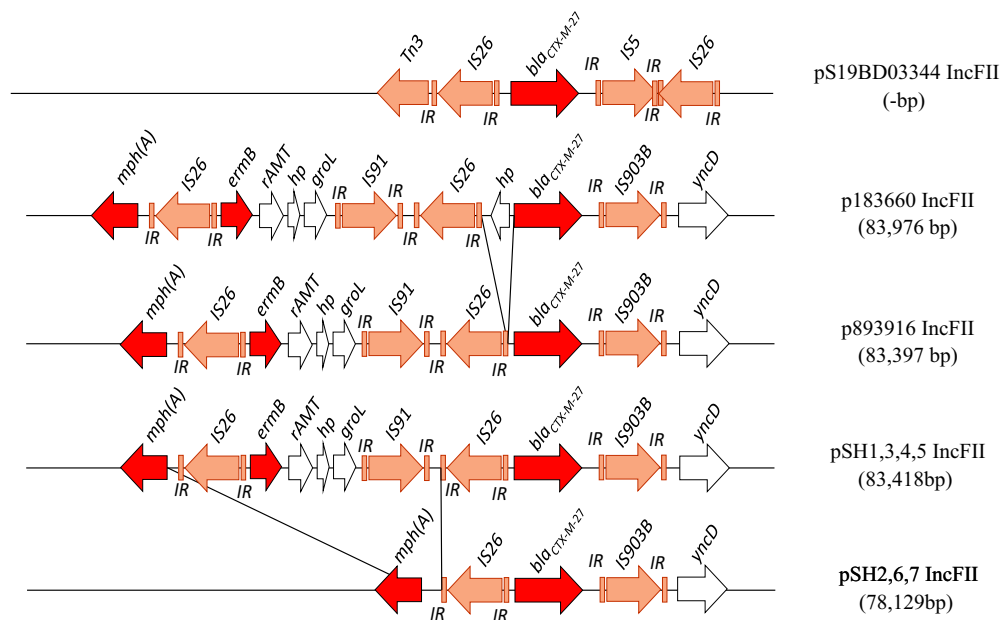
## Results and discussion

### Patients

Demographic data about the patients is shown in Table 1. All patients were adult males that presented at the health centre or emergency department reporting fever and gastrointestinal symptoms, especially diarrhoea. Five of them received antimicrobial treatment (two azithromycin, two ciprofloxacin and one ertapenem). All of the patients had a favourable outcome. Of six patients who reported sexual history, four were identified as MSM. None of the seven patients reported engaging in chemsex (sexualized use of recreational drugs) or using illicit drugs or travelling recently.

### Resistance determinants

The susceptibility profile of all seven *S. sonnei* isolates showed resistance to penicillins, cephalosporins, fluoroquinolones, cotrimoxazole and azithromycin. The assembled sequences



**Figure 2.** Comparison of the genetic environments of *bla*<sub>CTX-M-27</sub> from the plasmids pS19BD03344 (PRJEB40097), p183660 (KX008967.1), p893916 (MW396858.1), pSH1,3,4,5 (ERR9353303, ERR9353305, ERR9353306, ERR9353307) and pSH2,6,7 (ERR9353304, ERR9353308, ERR9353309) present in *S. sonnei* clinical isolates. Red arrows correspond to *bla*<sub>CTX-M-27</sub>, white arrows correspond to non-resistance proteins and orange arrows corresponds to mobile genetic elements (insertion sequence). IR, inverted repeat.

revealed several resistance determinants, including a *bla*<sub>CTX-M-27</sub> gene (responsible for the ESBL profile), a *qnrB19* gene and three mutations in the QRDRs: *gyrA* S83L, D87G and *parC* S80I, and azithromycin resistance genes [*ermB* and *mph(A)*]. Others resistance genes detected were *mdf(A)*, *dfrA*, *sul1*, *sul2*, *aadA5*, *aph(3'')-Ib*, *aph(6)-Id* and *tet(A)* (Table 1). Regarding the virulome, the *senB* gene, which encoded the *Shigella* enterotoxin (shET2), was detected in all the isolates. This gene is a major virulence factor in *S. sonnei*, responsible for the bacterial pathogenesis. Other important virulence factors such as *iucB* (aerobactin) and *sigA* (protease) were detected.

### Phylogenetic analysis

Samples SH1 (ERR9353303), SH3 (ERR9353305), SH4 (ERR9353306) and SH5 (ERR9353307) belong to the same cgMLST, 147566, according to the Enterobase *Escherichia/Shigella* cgMLST scheme, while samples SH2 (ERR9353304), SH6 (ERR9353308) and SH7 (ERR9353309) belong to cgMLST 174409 (Figure 1). All these isolates are within hierarchical cluster (HC) 5 98748 and therefore have less than five alleles of difference,<sup>11</sup> which indicates that they may belong to the same outbreak. These isolates were compared with others from two recent MSM outbreaks in Belgium,<sup>12</sup> the UK<sup>13</sup> and Australia,<sup>14</sup> and another single isolate from the Virgen Macarena University Hospital (Spain). Results of the analysis show that the isolate from Spain, two isolates from Australia and one from the UK belong to the same HC (HC5) as our isolates. Furthermore, genotyping according to Hawkey et al.<sup>15</sup> assigned the same genotype to all these isolates (3.6.1.1.2), belonging to clade 3.6 and lineage L3 (Figure 1, Table 1).

### Genetic context and epidemiology of *bla*<sub>CTX-M-27</sub>

All the strains carried a large IncFII plasmid (78 or 83 kb), which harboured the *bla*<sub>CTX-M-27</sub> gene. They demonstrated a higher similarity with the recently published plasmids p893916 and p183660 (99%–100% identity), which have been described among a collection of *S. sonnei* isolated in the UK.<sup>13</sup> Several outbreaks involving *S. sonnei* have been reported around the world and especially in Europe in the last decade.<sup>12,13,16,17</sup> Since those isolated carry the *bla*<sub>CTX-M-27</sub> gene, the surrounding sequences of this ESBL were compared with all available Belgium and UK sequences of CTX-M-27-producing *S. sonnei*, which would have been related with MSM transmission, to understand the mechanism of mobilization followed by the *bla*<sub>CTX-M-27</sub> resistance gene (Figure 2).

The *bla*<sub>CTX-M-27</sub> gene (876 bp) was flanked upstream by *IS26* and downstream by *IS903B* in all *S. sonnei* isolated in this study, presenting an identical structure in comparison with the isolates 893916 (2020) and 183660 (2015) from the UK, but different from the isolate S19BD03394 (2019) from Belgium.<sup>10</sup> All IncFII plasmids from the seven *S. sonnei* of this study harbour the previously described pKSR100 integron present in p183660 with sulphonamide, trimethoprim and aminoglycoside resistance genes (*sul1/dfrA17/aadA5*) alongside *emrE* (*qacEdelta1*), quaternary ammonium compound-resistance protein.<sup>11</sup> Instead, only four out of seven isolates contain the *mph(A)-ermB* unit accompanied by *IS91*. In SH2, SH6 and SH7 plasmids, the *IS26-ermB-rAMT-GroL-IS91* fragment from the *mph(A)-ermB* unit is missing (Figure 2). The different environment found in the flanked sequence of *bla*<sub>CTX-M-27</sub> together with the high proportion of *IS26* in the plasmid and especially near to the resistance genes suggested that the rapid reorganization and high plasticity of IncFII plasmids are likely driven by *IS26*.

PlasmidFinder also showed a large IncB/O/K/Z plasmid (86 kb) in all *S. sonnei* isolates with high nucleotide similarity (99.97% identity; 99% coverage) to a plasmid circulating in the UK (2020) (MW396864.1).<sup>11</sup> This plasmid lacked AMR determinants, implying that the functionality of the genes is sufficient to compensate any loss of fitness. Noticeably, SH1 and SH3 isolates also harboured a ColE1-like plasmid carrying genes for resistance to aminoglycosides [*aph(6)-I<sub>d</sub>* and *aph(3'')-I<sub>b</sub>*], sulphonamides (*sul2*) and tetracycline *tet(R)/tet(A)*, corresponding to the resistance determinants of the IncB/O/K/Z plasmids (JAENSM000000000 and JAEMECO000000000) recovered in the UK (2018) from *S. sonnei*.<sup>11</sup> This small plasmid showed high similarity (99.37%–99.95% identity; 99%–100% coverage) with several ColE1-like plasmid circulating in the USA [pCFSAN030807 (CP023647.1)], South Korea [pFORC11.3 (CP010832.1)], Italy [pLC1477\_18-3 (CP035011.1)] and India [pFC1653 (CP037998)]. Moreover, an additional IncFIB plasmid (109 kb) without resistance determinants in its sequence was found in SH2, SH6 and SH7 isolates that presented a high nucleotide similarity (99.98% identity; 98% coverage) to other plasmids recovered in Australia [pAUSMDU00008333\_02 (LR213459.1)] and the USA [pMHMC-002 (CP053753.1)].

## Conclusions

This study is in concordance with the ECDC warning since *S. sonnei* similar to extensively drug resistant strains circulating in Europe were isolated in Seville in late 2021. Furthermore, all our isolates belonged to the same outbreak and were closely related with the strains isolated in the UK and Belgium. Our results suggest that we are dealing with a high-risk clone of *S. sonnei* in continuous evolution. The differences in terms of plasmid structures as well as the number of plasmids harboured by the seven *S. sonnei* isolates seems to indicate that this outbreak was produced by the transmission of one clone that is able to evolve and disseminate rapidly. This could mean that the *S. sonnei* ST152 is a microorganism with a high niche-adaptive capacity, being able to coevolve with its host and respond to the selective pressure of its environment. Therefore, tracking the spread of successful epidemic clones of *S. sonnei* and understanding their evolution is important for the monitoring and control of such an international outbreak.

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## Transparency declarations

None to declare.

## Author contributions

A.R.-V. and J.A.L. conceived the study and designed the experiments, analysed the results and wrote the manuscript. J.M.O.R., C.S.C.-S., M.A.F., M.R.-P.P. and E.B. performed the experiments, analysed the results and wrote the manuscript. All the authors reviewed the manuscript.

## Supplementary data

Table S1 is available as [Supplementary data](#) at JAC-AMR Online.

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