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From farm to fork: Spread of a multidrug resistant *Salmonella* Infantis clone encoding *bla*$_{\text{CTX-M-1}}$ on pESI-like plasmids in Central Italy

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ABSTRACT

Salmonella enterica subsp. *enterica* serovar Infantis (*S.* Infantis) is one of the "top five *Salmonella* serovars" of clinical significance in the European Union (EU). Antimicrobial resistant and extended spectrum β-lactamase (ESBL) AmpC-producing *S.* Infantis have been described in food production systems and human clinical samples in Italy. Recently, an increase of MDR *S.* Infantis carrying *bla*$_{\text{CTX-M}}$ genes involved in 3rd generation cephalosporin resistance was noticed in the EU, including Italy, mainly due to the spread of *S.* Infantis harboring a pESI-like plasmid.

The aim of this study was to investigate the occurrence of the *S.* Infantis pESI-like plasmid among antibiotic resistant *S.* Infantis strains isolated at different points of the food chain, and to provide a phylogenetic analysis to gain further insight on their transmission pathways from 'farm to fork'.

MDR *S.* Infantis strains (n. 35) isolated from 2016 to 2021 at different stages of the food chain (animals, food, food-related environments, and humans) were investigated with in depth molecular characterization using real-time PCR, S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and whole genome sequencing (WGS).

Our study reported the occurrence of *S.* Infantis strains harboring pESI-like plasmids, carrying *bla*$_{\text{CTX-M-1}}$ genes, in Central Italy, at different sampling points along the food chain. Results confirmed the presence of a plasmid with a molecular size around 224–310 kb, thus consistent with the pESI-like, in 97 % of the 35 samples investigated. Two variants of *S.* Infantis pESI-like IncFIB(K)_1_Kpn3 were detected, one associated with the European clone carrying *bla*$_{\text{CTX-M-1}}$ (21 isolates) and the other associated with U.S. isolates carrying *bla*$_{\text{CTX-M-65}}$ (2 isolates, pESI-like U.S. variant). The majority was resistant to 3rd generation cephalosporins but none of the strains tested positive for the carbapenemase encoding genes. A total of 118 virulence genes were identified in isolates harboring the pESI-like plasmid. cgMLST and SNP-based analysis revealed the presence of one main cluster, composed by strains isolated from the environment, animals, food and humans.

The results of this investigation underline the importance of phylogenetic studies to monitor and understand pathogen and AMR spread in a One Health approach.

1. Introduction

Salmonella enterica subsp. *enterica* serovar Infantis (*S.* Infantis) is one of the five main serovars, causing human salmonellosis in the European Union (EU). In 2021, in the same area, according to the latest report by EFSA (EFSA, 2023), the presence of multi-drug resistant (MDR) *S.* Infantis in broilers, turkeys and laying hens reached 83.9 %, 88.8 % and 24.5 % respectively. Moreover, in 2021, 38 % of *S.* Infantis isolated in the EU from human clinical samples were MDR (EFSA, 2023).

The issue of MDR in food production systems is particularly concerning where it involves critically important antibiotics (CIAs) (WHO, 2019) for human health. In the last decade, MDR and extended spectrum β-lactamase (ESBL) AmpC-producing *S.* Infantis have been frequently reported from food-producing animals and humans in Italy (Food and

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Authority, 2022; Di Marcantonio et al., 2022).

Indeed, there is a high prevalence of the MDR ESBL phenotype in Italian isolates from broiler and turkeys, some of which also carry the AmpC profile (Food and Authority, 2022), although the use of 3rd and 4th generation cephalosporin is not permitted in poultry and off-label use is prohibited according to EU legislation (Food and Authority, 2022; Franco et al., 2015a; EMA, 2009).

Thus, the high proportion of MDR ESBL/AmpC-producers in *S.* Infantis can be attributed to a clonal expansion and spread, as described by Franco et al. (Franco et al., 2015a). One example for this spread is a particular MDR and ESBL producing *S.* Infantis clone, which carries an IncI/IncP chimeric megaplasmid (~280–320 kb), encompassing virulence, fitness and MDR genes, with emphasis on the ESBL encoding gene $bla_{\text{CTX-M-1}}$. This megaplasmid is named pESI-like since it is similar to that described in Israel in 2014 isolated from *S.* Infantis (Aviv et al., 2014). Initially, pESI was isolated in Israel and contributes to tetracycline, sulfamethoxazole and trimethoprim resistance in *S.* Infantis extended-spectrum cephalosporin sensitive (ESC-S) (Franco et al., 2015a; Aviv et al., 2014). Moreover, the pESI plasmid was found to promote pathogenicity mechanisms and was described to be associated with the increase of intestinal inflammation in experimental mouse infections. The pESI from Israel also harbored genes enabling an increase in bacterial tolerance to environmental mercury (*mer* operon) and oxidative stress. (Franco et al., 2015a; Aviv et al., 2014). Later, a plasmid similar to that described in Israel was isolated from emerging *S.* Infantis lineages worldwide and named pESI-like. This plasmid displayed the same backbone pESI, but in addition carried antibiotic resistant ESBL genes (Cohen et al., 2022).

The *S.* Infantis clone harboring the pESI-like plasmid was identified in Italy by Franco et al. from food-producing animals and humans (Franco et al., 2015a), and it has increasingly spread during the last years (Alba et al., 2020a).

This pESI-like megaplasmid is characterized by a specific gene panel that includes genes conferring resistance against 3rd generation cephalosporins ($bla_{\text{CTX-M-1}}$), tetracyclines (*tet*A), trimethoprim (*dfr*A), sulphonamides (*sul*1), aminoglycosides (*aad*A). Moreover, other advantageous and virulence-enhancing genes associated to pESI-like plasmids include yersiniabactin biosynthetic protein gene *irp*2, fimbria gene *ipf*, toxin/antitoxin (T/AT) system and genes conferring resistance to quaternary ammonium compounds (Alba et al., 2020a).

Due to its previously described antibiotic resistance genes (ARGs), genes involved in virulence, colonization and fitness, pESI-like plasmid, once acquired, has become established in the local population thanks to the selective advantages it confers to the host bacteria (Alba et al., 2020a; Vázquez et al., 2022).

The *S.* Infantis pESI-like carrying $bla_{\text{CTX-M-1}}$ gene variant, with all the previously described features, was usually isolated in the EU (Alba et al., 2020a). On the other hand, in the United States (U.S.), in 2018, an outbreak of an ESBL-producing *S.* Infantis which harbors a pESI-like megaplasmid that carries $bla_{\text{CTX-M-65}}$, associated with raw chicken, was reported. The majority of the strains harboring $bla_{\text{CTX-M-65}}$ were additionally resistant to ciprofloxacin, nalidixic acid, chloramphenicol, gentamicin, sulfamethoxazole, tetracycline and trimethoprim (Food and Authority, 2022; McMillan et al., 2020). Moreover, pESI-like described largely in the U.S. has integrated a larger region including $bla_{\text{CTX-M-65}}$ together with other resistance genes towards antibiotics (*e.g. floR*, *fosA*, *aph(4)-Ia*) or heavy metals (*arsH*) (McMillan et al., 2022; Alba et al., 2021). In this regard, pESI-like $bla_{\text{CTX-M-65}}$-positive *S.* Infantis isolated in the EU were associated to travel history to Asia and South America, indeed this "variant" did not appear to be associated with the European *S.* Infantis (Alba et al., 2021).

The ESBL-producing *Enterobacterales* are defined "Critical Priority Pathogens" by the World Health Organization (WHO), since they show resistance to 3rd generation cephalosporin considered as CIA for human health and first choice treatment for children and invasive infections (Franco et al., 2015a; Badescu et al., 2022; Critically Important Antimicrobials for Human Medicine 6th Revision, 2018). Due to these characteristics and the ability to spread and establish well in the host cell, this plasmid was described to be able to spread in the way 'from farm to fork' (Alba et al., 2020a).

The aim of this study was to investigate the occurrence of the *S.* Infantis pESI-like plasmid among antibiotic resistant *S.* Infantis strains isolated at different points of the food chain (primary production: animals and environment; food; food processing plants; humans) in Marche Region, Central Italy, and to study their genetic characteristics and AMR profiles. Phylogenetic analysis of *S.* Infantis strains and associated pESI-like plasmids were conducted to gain further insight on their transmission pathways from 'farm to fork'.

2. Materials and methods

2.1. S. Infantis strains

For this study, 35 AMR *S.* Infantis strains isolated during routine surveillance by the Regional Reference Center for Enteric Pathogens of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, section of Tolentino, were analyzed. Strains were isolated from 2016 to 2021, at different stages of the food chain, from food (6 from chicken meat, 1 from pig meat, 1 from mollusks and 3 from mixed meat samples); food-related environment (3 from livestock, 1 from slaughterhouse and 1 from food processing laboratory); animals (8 from broiler) and humans (10 from faeces and 1 from other human clinical sample). The AMR profiles of *S.* Infantis strains included in this study had been previously investigated by us, through phenotypic assays, as described in the paper by Russo et al. (Russo et al., 2022). In detail, the antimicrobial susceptibility of the *Salmonella* strains was determined by the disk diffusion method, according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2022), and all isolates were found to be resistant to three or more antibiotic classes (MDR profile) (Magiorakos et al., 2012) (Table S1).

Strain identity, at the genus level, was confirmed through real-time PCR by using a protocol previously published by us (Magiorakos et al., 2012). Briefly, bacterial DNA obtained by colony lysis by boiling was used as template in a real-time PCR assay targeting a 155 bp sequence of the genus-specific *ttr*C gene, with ttr1 (GCAGGAGGTCTGAATGACG) and ttr2 (TTTTCCGCCAGTGAAGATAAC) primers (0.6 μM each) and P25 (ACTCATCATTGAAGAAGTGCTGGCTCAC) 5' FAM labelled probe (0.1 μM), with the following thermal protocol: denaturation at 95 °C 10 min; 30 cycles at 95 °C 20 s and 63 °C 1 min (Magiorakos et al., 2012).

2.2. Real-time PCR screening for the presence of genes conferring resistance against 3rd generation cephalosporins and carbapenems

2.2.1. DNA extraction via thermal lysis

Bacterial isolates were grown on Trypticase soy agar (TSA) plates overnight. A single colony was resuspended in 300 μL of TE and heated to 100 °C for 10 min. Afterwards the suspension was pelletized at 14,000 rpm for 2 min and 50 μL of supernatant was added to 100 μL of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) to dilute the final concentration of DNA. The DNA was stored at −20 °C and used for PCR detection of carbapenemases and ESBL/CIT-type AmpC encoding genes.

2.2.2. Detection of carbapenemase and ESBL/CIT-type pAmpC encoding genes by multiplex real-time PCR

The extracted DNA was used as template in two real-time PCR assays, one for the detection of genes encoding ESBL and AmpC enzymes and the other targeting carbapenemase genes.

The detection of ESBL and Amp-C encoding genes was carried out *via* multiplex real-time PCR for the identification of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and CIT-Type AmpC genes (Roschanski et al., 2014).

Multiplex real-time PCR was performed in 20 μL, including PCR mastermix (Platinum Multiplex PCR Master Mix, Thermo Fisher,

Waltham, MA, USA) and 1 μL of boiled DNA. Sequences of primers and probes with final concentrations are listed in Table S2 (Roschanski et al., 2014). The PCRs were performed with an initial denaturation step of 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 5 s, annealing and elongation at 60 °C for 60s.

A further multiplex real-time PCR was carried out for the detection of the most frequent carbapenemase genes bla_{VIM}, bla_{KPC}, bla_{NDM}, bla_{OXA-48}, and bla_{GES}. Primer and probe sequences for this assays were based on previously published protocols (Swayne et al., 2011; Van der Zee et al., 2014), which were later adapted by Roschanski et al. (Roschanski et al., 2017). Complete sequences with final concentrations are described in Table S3 (Roschanski et al., 2017).

Multiplex real-time PCR was performed in 25 μL with Platinum Multiplex PCR Master Mix and 1 μL of boiled DNA. The amplification reactions were performed with an initial denaturation step of 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 15 s, annealing and elongation at 60 °C for 60 s.

2.3. Whole genome sequencing (WGS)

The 35 *S. infantis* strains were cultivated in TSA. A single colony of each strain was inoculated in Luria-Bertani (LB) liquid medium and incubated at 37 °C with overnight shaking conditions.

The PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) was used for Genomic DNA extraction.

Sequencing libraries were prepared with the Nextera™DNA Flex Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Paired-end sequencing was performed in 2 × 149 cycles on the Illumina NextSeq™ 500 benchtop sequencer (Illumina, San Diego, CA, USA) using the NextSeq™ 500/550 Mid Output Kit v2 or v2.5 (300-cycle) (Illumina, San Diego, CA, USA).

The bioinformatics AQUAMIS pipeline v1.3.8 (Assembly-based Quality Assessment for Microbial Isolate Sequencing) was used for the assembly and trimming of the raw reads (Deneke et al., 2021a) (https://gitlab.com/bfr_bioinformatics/AQUAMIS). Based on the draft assemblies produced by AQUAMIS, the BakCharak pipeline v2.1.0 was used to conduct a characterization of the *Salmonella* genomes (https://gitlab.com/bfr_bioinformatics/bakcharak). The BakCharak pipeline implements the following tools: ncbi-amrfinderplus v3.9.3 to detect antimicrobial resistance genes (Feldgarden et al., 2019); PlasmidFinder database for plasmid incompatibility group detection (Carattoli et al., 2014); ABRicate v1.0.1 for antimicrobial resistance and virulence factor screening (https://github.com/tseemann/abricate); Prokka 1.14.6 for gene annotation; AMR finder 2021-03-01.1; mlst 2.19.0 for ST typing. *Salmonella* serovar is determined with software SISTR v1.1.1 (Yoshida et al., 2016).

Twelve strains, representative of all *S. infantis* isolates investigated in this study, were selected based on isolation source, AMR characteristics and plasmid content for sequencing by Oxford Nanopore MinION technology (Table 1). ONT libraries were prepared using the Rapid barcoding kit 96 (SQK-RBK110-96, Oxford Nanopore Technologies, Oxford, UK). Libraries were sequenced for approximately 48 h on a MinIon Mk1C device (running on MinKNOW v21.11.6) using a FLO-MIN106 R9.4.1 flow cell. For long-read assembly, the assembly software flye (version 2.9) (Walker et al., 2014) was used and the generated assemblies were subsequently polished with Illumina data using pilon (5 iterations) (Kolmogorov et al., 2019). Moreover, long read sequencing allowed to confirm the location of AMR genes, virulence genes and pESI-like gene pattern on the plasmid.

The resulting long-read assemblies were analyzed using the BakCharack pipeline to verify the plasmidial location of the bla_{CTX-M} genes and to confirm their co-localization with pESI-like gene patterns. In addition, the determined plasmid sizes were compared with the results of the S1-PFGE analysis.

2.4. Phylogenetic analysis

A cgMLST analysis including all *S.* Infantis strains was carried out for pre-clustering and to explore the genetic diversity of the isolate set to optimize subsequent SNP-based phylogenetic analysis.

The cgMLST analysis was conducted with the chewieSnake pipeline v3.1.1 (Deneke et al., 2021b), which implements chewBBACA v2.0.12

Table 1
S. Infantis strains sequenced using Oxford nanopore MinION technology, selected on the basis of isolation matrix, AMR content and plasmid replicon content, detected by WGS.

Samples ID	Isolation matrix	WGS AMR content	Plasmid replicon	Accession number[a]
22-SA00392-0	Food	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852512
22-SA00393-0	Food	blaCTX-M-1;gyrA_D87G;mdsA;mdsB	IncFIB(K)_1_Kpn3, IncFIC(FII)_1	SAMN37852513
22-SA00397-0	Environment	blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852517
22-SA00401-0	Environment	aph(3′)-Ia;blaCTX-M-1;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852521
22-SA00405-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852525
22-SA00406-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3; IncX4_1	SAMN37852526
22-SA00409-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852529
22-SA00449-0	Human	aac(3)-IVa;aadA1;aph(3′)-Ia;aph(4)-Ia; blaCTX-M-65;dfrA14;floR;gyrA_D87Y; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852533
22-SA00453-0	Human	blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852536
22-SA00454-0	Human	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852537
22-SA00455-0	Human	aac(3)-IVa;aadA1;aph(3′)-Ia;aph(4)-Ia; blaCTX-M-65;dfrA14;floR;fosA3;gyrA_D87Y; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852538
22-SA00456-0	Human	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852539

[a] Sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the Bioproject accession number PRJNA1028803.

(Silva et al., 2018) and using a 3000 loci scheme derived from Enterobase (http://enterobase.warwick.ac.uk/species/senterica/download_data). Allele profiles were generated and pairwise allele differences calculated. An allelic distance matrix was generated, and a minimum spanning tree calculated using GrapeTree (https://enterobase.warwick.ac.uk/ms_tree?tree_id=23518).

A threshold with a maximum cut-off of 20 allelic differences was used to group strains into clusters.

cgMLST results were also used to select appropriate references for the subsequent SNP analyses. 22-SA00401-0, belonging to cgMLST cluster 1, was selected as internal reference for SNP analysis on the bases of AMR profile, plasmid replicon profile, and pESI-like gene pattern profile.

On the other hand, the assembled sequence of plasmid 22-SA00401-0 was used as the reference for the analyses of the plasmid.

SNP calling was conducted with the pipeline snippySnake v1.0, which implements snippy v4.6.0 for variant calling (Lüth et al., 2021). A maximum-likelihood based phylogenetic tree was inferred with IQ-TREE v2.0.3 from the core SNPs of both, the chromosome and the plasmid. Phylogenetic trees were visualized with iTOL v5.

Single-linkage clustering was performed on the pairwise SNP difference between all isolates using a distance threshold of 23 SNPs (Pightling et al., 2018).

2.5. PFGE-S1 plasmids size characterization

PFGE analysis of S1 digested DNA was performed to confirm the presence and molecular sizes of plasmids. Analyses were conducted with the CHEF-DR III system (Bio-Rad Laboratories GmbH, Munich, Germany) using a 1 % agarose gel (Biozyme LE GP agarose; Biozym Scientific GmbH, Hessisch Oldendorf, Germany) under the following running conditions: 1 s–25 s, 17 h, 6 V/cm, 120 V, according to the PulseNet standardized laboratory protocol (Rodríguez et al., 2009). Plasmids sizes were evaluated using the molecular size marker *Salmonella enterica* serovar Braenderup H9812 digested with *Xba*I enzyme.

2.6. pESI-like prediction

Trimmed reads of the 35 *S.* Infantis strains were aligned against reference sequences of 13 target genes proposed by McMillan et al. (McMillan et al., 2020) (Table S4), indicative of pESI-like plasmid presence, using Geneious Prime® 2020.2.2. Isolates carrying genes with >95 % identity to the target sequences were considered positive for the presence of the gene (McMillan et al., 2020). Moreover, for genes with a coverage <95 % to the target sequences, short read sequences of *S.* Infantis strains were additionally aligned *in silico* using Geneious Prime® 2020.2.2, using PCR primers proposed by Franco et al. to verify the presence of the gene (Franco et al., 2015b).

Geneious Prime® was used to carry out the mapping between 12 long read plasmid sequences and pESI-like genes sequences proposed by McMillan et al. (McMillan et al., 2020).

3. Results

3.1. Presence of genes encoding for 3rd generation cephalosporin or carbapenem resistance in MDR S. Infantis isolates

The AMR profiles of *S.* Infantis strains included in this study, previously investigated by our research group, showed a total of 74 % (26/35) of those strains were resistant to at least one beta-lactam and, among them, 88 % (23/26) were resistant to 3rd generation cephalosporins (Russo et al., 2022).

Real-time PCR analysis showed 88 % of isolates were positive for $bla_{\text{CTX-A/B}}$ gene family, 12 % for bla_{TEM} gene family. The only strain with an intermediate resistance for CTX showed the absence of $bla_{\text{CTX-A/B}}$ gene family.

None of the strains tested positive for one of the carbapenemase encoding genes investigated in this study.

3.2. Sequence analysis of S. Infantis strains

3.2.1. Gene content

BakCharak based sequence analyses of the 35 *S.* Infantis strains revealed two different $bla_{\text{CTX-M}}$ variants: 60 % (21/35) of strains carried $bla_{\text{CTX-M-1}}$ and 6 % (2/35) $bla_{\text{CTX-M-65}}$, both involved in 3rd generation cephalosporin resistance. Eight percent (3/35) carried $bla_{\text{TEM-1}}$ and 26 % (9/35) were negative for all ESBL/AmpC genes.

The most frequently detected genes involved in AMR were: *tet*A, *dfr*A1, and *dfr*A14 variants, which were carried by 97 % (34/35) of strains, followed by *sul*1, 94 % (33/35), and *aph*(3′), 40 % (14/35). Additional genes included *aad*A1, *ant(2″)*, *mdsA* and *mdsB*. Notably, *aac*(3)-IVa, *aph*(4), *floR* and *fosA3* genes were found only in $bla_{\text{CTX-M-65}}$ positive strains. A mutation in the *gyr*A gene, usually associated with fluoroquinolone resistance (D87G variant), was found in all strains. Moreover, long read sequencing confirmed the position of all AMR genes on the plasmid except for *gyr*A genes, *mdsA* and *mdsB*. All ARGs are listed in Table 2.

A total of 118 virulence genes were identified in isolates harboring the pESI-like plasmid, including genes encoding fimbriae, *feaD*, *feaE*, yersiniabactin operon genes *fyuA*, *irp*1, *irp*2, *ybt*A, *ybt*E, *ybt*P, *ybt*Q, *ybt*S, *ybt*T, *ybt*U, *ybt*X, mercury operon involved in mercury resistance such as *mer*A gene. In the strains without the plasmid, 105 virulence genes were identified (Table S5). Genes missing in strains without plasmids were: *fae*D, *fae*E, *fyu*A, *irp*1, *irp*2, *ybt*A, *ybt*E, *ybt*P, *ybt*Q, *ybt*S, *ybt*T, *ybt*U, *ybt*X. In particular, after the analysis of twelve long read sequences, the following virulence genes were detected on the plasmid: *fae*D, *fae*E, *fyu*A, *irp*1, *irp*2, *ybt*A, *ybt*E, *ybt*P, *ybt*Q, *ybt*S, *ybt*T, *ybt*U, *ybt*X.

3.2.2. Plasmid content

The investigation of plasmid replicon profiles revealed 97 % (34/35) of *S.* Infantis isolates possessing the incompatibility group (Inc) FIB(K)_1_Kpn3, which was present also in strains lacking $bla_{\text{CTX-M-1}}$ (Table 2). In eight strains, IncFIB(K)_1_Kpn3 was observed together with other incompatibility groups. The second most common Inc. group, found in four isolates, was IncX4, followed by IncX1 detected in three isolates, and X3, along with FII and FIC, present in one isolate each. The Col (MG828)_1 replicon was found in two strains (Table 2).

3.2.3. Presence of pESI-like gene pattern

All 35 *S.* Infantis strains were screened for the pESI-like target sequences to predict the presence of the plasmid. As shown in Table 3, *S.* Infantis strains showed a total or partial presence of the pESI-like gene pattern. In particular, genes *ard*A, I1 relaxase, *sog*S, *trb*A, K88, Yersinia bactime operon were found in 97 % (34/35) strains, *ipf* in 100 % of strains and *pilL* in 94 % (33/35) strains. The replication origin *rep*B was associated with the IncFIB(K)_1_Kpn3 plasmid. IncP was found in 94 % (33/35) strains, pESI backbone in 91 % (32/35), *mer*A in 94 % (33/35).

The pESI backbone target was not observed in the two pESI-like $bla_{\text{CTX-M-65}}$ harboring isolates.

IncP (iterons associated with IncP plasmids), *mer*A and *ipf* were also detected in strains that did not carry the IncFIB(K)_1_Kpn3 plasmid.

3.2.4. Alignment of short read sequences

The alignment of *S.* Infantis short read sequences to pESI-like reference genes revealed that all target genes showed >95 % of identity except for the *sog*S gene, which featured an identity of 94.2 %.

To verify the real presence of the *sog*S gene, strains sequences were aligned against the *sog*S gene primers proposed by Franco et al. (Franco et al., 2015a). This confirmed the presence of the gene in all strains harboring IncFIB(K)_1_Kpn3 plasmid *via in silico* PCR.

The $bla_{\text{CTX-M-1}}$ was the most detected gene among those conferring resistance to 3rd generation cephalosporin, followed by the $bla_{\text{CTX-M-65}}$

Table 2
AMR and plasmid replicon profiles obtained by WGS. Bakcharak was used to carry out the analysis.

Sample ID	Sources	AMR profile	Plasmid replicon profile	Accession number[a]
22-SA00386-0	Food	aadA1;aph(3′)-Ia;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852506
22-SA00387-0	Food	aph(3′)-Ia;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852507
22-SA00388-0	Food	aph(3′)-Ia;blaTEM-1;dfrA14; gyrA_D87G;mdsA;mdsB;tet(A)	IncFIB(K)_1_Kpn3; IncX4_1	SAMN37852508
22-SA00389-0	Food	aadA1;blaCTX-M-1;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3; IncX1_1	SAMN37852509
22-SA00390-0	Food	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852510
22-SA00391-0	Food	aph(3′)-Ia;blaCTX-M-1;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3; IncFII(29)_1_pUTI89	SAMN37852511
22-SA00392-0	Food	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852512
22-SA00393-0	Food	blaCTX-M-1;gyrA_D87G;mdsA;mdsB	IncFIB(K)_1_Kpn3, IncFIC(FII)_1	SAMN37852513
22-SA00394-0	Food	dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852514
22-SA00395-0	Food	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852515
22-SA00396-0	Food	aph(3′)-Ia;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852516
22-SA00397-0	Environment	blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852517
22-SA00398-0	Environment	blaCTX-M-1;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852518
22-SA00399-0	Environment	aph(3′)-Ia;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3; IncX4_1	SAMN37852519
22-SA00400-0	Environment	aph(3′)-Ia;blaCTX-M-1;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852520
22-SA00401-0	Environment	aph(3′)-Ia;blaCTX-M-1;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852521
22-SA00402-0	Animal	aph(3′)-Ia;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	Col(MG828)_1; IncFIB(K)_1_Kpn3	SAMN37852522
22-SA00403-0	Animal	aph(3′)-Ia;dfrA1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3; IncX1_1;IncX3_1	SAMN37852523
22-SA00404-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852524
22-SA00405-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852525
22-SA00406-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3; IncX4_1	SAMN37852526
22-SA00407-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852527
22-SA00408-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852528
22-SA00409-0	Animal	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852529
22-SA00446-0	Human	ant(2″)-Ia;blaTEM-1;cmlA5;dfrA1; gyrA_D87Y;mdsA;mdsB;sul1;tet(A)		SAMN37852530
22-SA00447-0	Human	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852531
22-SA00448-0	Human	dfrA1;gyrA_D87G;mdsA; mdsB;sul1;tet(A)	Col(MG828)_1; IncFIB(K)_1_Kpn3	SAMN37852532
22-SA00449-0	Human	aac(3)-IVa;aadA1;aph(3′)-Ia;aph(4)-Ia; blaCTX-M-65;dfrA14;floR;gyrA_D87Y; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852533
22-SA00450-0	Human	aadA1;aph(3′)-Ia;blaTEM-1;dfrA14; gyrA_D87G;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3; IncX1_1;IncX4_1	SAMN37852534
22-SA00451-0	Human	blaCTX-M-1;dfrA1;gyrA_D87G; gyrA_S83Y;mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852535
22-SA00453-0	Human	blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852536
22-SA00454-0	Human	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852537
22-SA00455-0	Human	aac(3)-IVa;aadA1;aph(3′)-Ia;aph(4)-Ia; blaCTX-M-65;dfrA14;floR; fosA3;gyrA_D87Y; mdsA; mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852538
22-SA00456-0	Human	blaCTX-M-1;dfrA1;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852539
22-SA00457-0	Human	aph(3′)-Ia;dfrA1;dfrA14;gyrA_D87G; mdsA;mdsB;sul1;tet(A)	IncFIB(K)_1_Kpn3	SAMN37852540

[a] Sequencing data for isolates of this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the Bioproject accession number PRJNA1028803.

Table 3
Presence and absence of target genes used to predict the presence of pESI-like plasmid in the 35 *S.* Infantis isolates, analyzed in this study. Strains sequenced using MinION technology were also reported.

	ardA	I1 relaxase	sogS	trbA	IncP	pESI backbone	K88(fae)	Ybt	mer A	ipf	pilL	repB	blaCTX-M-1	blaCTX-M-65	blaTEM-1	IncFIB(K)	MinION
22-SA00386-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00387-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00388-0	+	+	+	+		+	+	+		+	+	+			+	+	
22-SA00389-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00390-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00391-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00392-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00393-0	+	+	+	+		+	+	+		+		+	+			+	x
22-SA00394-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00395-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00396-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00397-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00398-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00399-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00400-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00401-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00402-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00403-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00404-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00405-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00406-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00407-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00408-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00409-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00446-0					+				+	+					+		
22-SA00447-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00448-0	+	+	+	+	+	+	+	+	+	+	+	+				+	
22-SA00449-0	+	+	+	+	+		+	+	+	+	+	+		+		+	x
22-SA00450-0	+	+	+	+	+	+	+	+	+	+	+	+			+	+	
22-SA00451-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
22-SA00453-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00454-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00455-0	+	+	+	+	+		+	+	+	+	+	+		+		+	x
22-SA00456-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+	x
22-SA00457-0	+	+	+	+	+	+	+	+	+	+	+	+				+	

variant.

3.2.5. Confirmation of pESI-like gene location on plasmid using long read sequence data

The mapping of twelve plasmid sequences, obtained using MinION technology, against pESI-like gene pattern sequences confirmed the location of these genes on the plasmid (Table 3).

Out of all isolates, 26 % (9/35) possessed the IncFIB(K)_1_Kpn3 plasmid, without carrying one of the ESBL encoding genes.

The only strain (22-SA00446-0) lacking plasmid showed the presence of three pESI-like gene targets: IncP, *mer*A and *ipf*.

3.3. Results of plasmids size determination via S1-PFGE

S1-PFGE analysis of the 35 *S.* Infantis strains confirmed the presence of plasmids with 224–310 kb size in 34/35 strains and the absence of any plasmid in one isolate (22-SA00446-0). The presence of other plasmids was also confirmed in ten isolates. Six isolates carrying genes of IncX incompatibility group also showed additional bands of 33 kb in S1-PFGE. Moreover, two isolates, which showed presence of IncFIC and IncFII in Bakcharak results, possessed an additional band of around 55 kb.

In two strains the presence of additional Col(MG828)_1 plasmids, that were observed *via* WGS analysis, could not be confirmed with S1-PFGE.

3.4. cgMLST analysis

cgMLST analysis revealed the presence of one main cluster, further referred to a maximum distance matrix of 31 (Cluster 1), composed by 29 strains isolated from the environment, animal, food and humans.

The majority of strains belonged to the same Cluster 1, with a maximal pairwise genetic distance of 20 alleles. The minimum distance found was one allelic difference between one environmental strain and one animal strain, both negative for the $bla_{\text{CTX-M-1}}$ gene. The minimal AD between a human strain and an animal strain, both carrying $bla_{\text{CTX-M-1}}$, was three.

Six strains did not belong to cluster 1 and did not form further clusters: in two of those IncFIB(K)_1_Kpn3, IncX4 and IncX1 plasmids were detected, and $bla_{\text{TEM-1}}$ were found; two harbored IncFIB(K)_1_Kpn3 plasmid probably carrying $bla_{\text{CTX-M-65}}$. Finally, in one isolate any plasmid could be observed but a $bla_{\text{TEM-1}}$ gene.

The largest allelic distance of 93 AD was found among strains outside cluster 1, between the only strain without a plasmid, isolated from a clinical sample, and a food isolate harboring $bla_{\text{TEM-1}}$.

Excluding the strains without any plasmid, the largest AD was 88, and it was detected between one strain carrying $bla_{\text{CTX-M-65}}$ and one strain carrying $bla_{\text{TEM-1}}$ (distance matrix in Supplementary Fig. S1).

3.5. SNP analysis

SNP analysis, based on the sequences of 35 *S.* Infantis strains, revealed a variation ranging from 1 to 314 SNPs using the internal reference 22-SA00401-0. 22-SA00401-0 was selected as internal reference, because it belonged to the main cgMLST Cluster 1 and is the oldest strain harboring a pESI-like plasmid carrying $bla_{\text{CTX-M-1}}$ with a complete pESI-like gene pattern (Table 3).

Minimum SNPs difference was one SNP between two strains isolated from animal sources and four SNPs between two strains isolated from different sources (one from animal and one from human).

The maximum SNPs distance between one strain carrying $bla_{\text{TEM-1}}$ and the only strain without plasmid was 314. Excluding the only strain without any plasmid, the maximum SNP distance was 286 between the same $bla_{\text{TEM-1}}$ strain and the strain carrying $bla_{\text{CTX-M-65}}$. These results confirm cgMLST analysis results, showing the maximum AD for the same strains.

According to Pightling et al., that hints to a suitable SNP threshold <21, we decided to define strains with SNP minimum distance matrix of 23 as part of the main SNP Cluster (cluster S1) (Fig. 1) (Pightling et al., 2018). In particular, 83 % (29/35) of *S.* Infantis strains were assigned to the Cluster S1 based on the SNP threshold of 23. Notably, this cluster contains strains harboring IncFIB(K)_1_Kpn3 plasmids with or without a $bla_{\text{CTX-M-1}}$ gene. Out of the six isolates outside of the SNP-Cluster S1, three carried a $bla_{\text{TEM-1}}$ gene and one of them did not carry any plasmid, two carried a $bla_{\text{CTX-M-65}}$ and one strain carried pESI-like plasmid without ESBL/AmpC genes.

The SNP cluster S1 could be divided into six subclusters, depicted in Table 4, with strains isolated from different sources, using a SNPs distance of ≤10. Moreover, sub-cluster 4 highlighted the presence of strains isolated in different steps along the meat production chain from livestock to food processing laboratory.

The SNP analysis of plasmids, using also 22-SA00401-0 as reference, showed a maximum SNP distance matrix of 18 among all IncFIB plasmids detected in this study. Moreover, all plasmids showed the maximum SNP distance, between 14 and 18 SNPs, against the two plasmids carrying $bla_{\text{CTX-M-65}}$ and representative of the pESI-like U.S. variant (Fig. 2).

4. Discussion

S. Infantis is an epidemiologically relevant pathogen for human salmonellosis (The European Food Safety Authority, 2021). In the last years, an increase of MDR *S.* Infantis carrying $bla_{\text{CTX-M}}$ genes involved in 3rd generation cephalosporin resistance was noticed in the EU, including Italy (Food and Authority, 2022), mainly due to the spread of *S.* Infantis harboring a pESI-like plasmid (Franco et al., 2015a).

In the present study, we demonstrated the presence of *S.* Infantis pESI-like in Central Italy (Marche Region) within strains isolated at different stages of the food chain (primary production: animals and environment; food; food processing plants; humans). Moreover, an in-depth molecular characterization revealed potential correlations between the strains and their plasmids.

The presence of plasmids with a molecular sizes around 224–310 kb, thus compatible with the pESI-like plasmid, was confirmed by the S1-PFGE in 97 % (34) of the 35 samples investigated. WGS short read data allowed the identification of the pESI-like gene pattern to predict presence of the pESI-like plasmids in those strains and long read data were used to confirm location of the genes on the plasmids. In fact, two variants of *S.* Infantis pESI-like IncFIB(K)_1_Kpn3 were detected, one associated with the European clone carrying $bla_{\text{CTX-M-1}}$ (Franco et al., 2015a) in 21 isolates and the other associated with U.S. isolates carrying $bla_{\text{CTX-M-65}}$ (McMillan et al., 2020) in two isolates.

Despite the presence of the $bla_{\text{CTX-M-1}}$ gene in the majority of pESI-like plasmids in the EU (Alba et al., 2020b), the absence of this gene is not associated with the absence of the plasmid (Franco et al., 2015a; Alba et al., 2020a; McMillan et al., 2020). Within the 34 *S.* Infantis pESI-like strains not all of them carried $bla_{\text{CTX-M}}$. The absence of this gene could be explained with: (i) the loss of the gene, due to the low exposition to the antibiotic (McMillan et al., 2020); (ii) the association of the $bla_{\text{CTX-M}}$ gene with transposable elements that allow its loss and acquisition though transposition of the gene even within different strains, also of different species, improving the dissemination of 3rd generation cephalosporin resistance (Cantón and Coque, 2006).

All target genes used to predict the presence of pESI-like plasmid in our isolate selection showed an identity <95 %, with the exception of *sog*S, which showed high identity only in pESI-like $bla_{\text{CTX-M-65}}$ but a slightly decreased identity of 94 % in 21 $bla_{\text{CTX-M-1}}$ harboring isolates. Probably in the Italian clone, this gene has different mutations compared to the sequence proposed by Mc Millan (McMillan et al., 2020), underlining the divergent evolution from the U.S. clone of Italian strains. However, to confirm the presence of the *sog*S gene, which showed lower identity (<95 %), the gene region was aligned with the

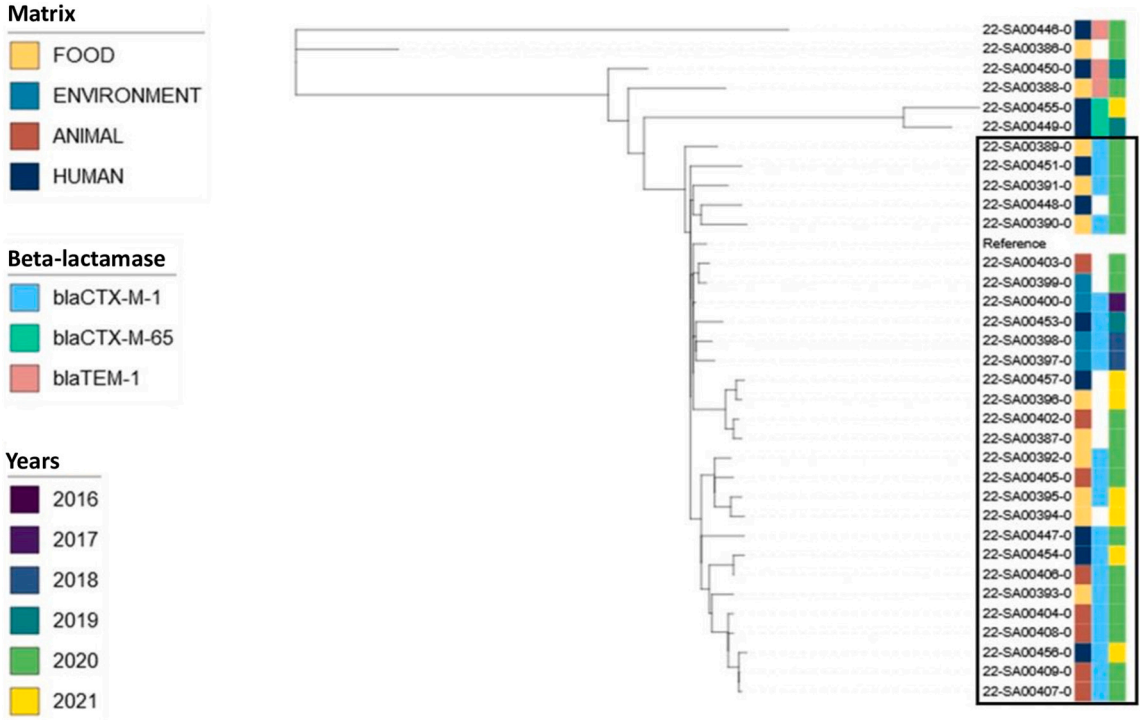

Fig. 1. SNP-based phylogenetic tree based on the WGS. The figure shows the phylogenetic relation between the 35 *S.* Infantis strains investigated. Colors near strain codes refer to isolation source, ESBL content and year of isolation. Strains included in the black box belong to the same cluster with a SNP minimum distance matrix of 23.

Table 4
S. Infantis strains of SNP cluster S1, divided according to the sub-cluster association defined by a SNP threshold of ≤10.

Code	Subcluster	Matrix	Year	IncFIB(K)	bla_{CTX-M}
22-SA00387-0	1	food:meat-based bovine products	2020	+	–
22-SA00402-0	1	animal: broiler	2020	+	–
22-SA00396-0	2	food:meat-based bovine products	2021	+	–
22-SA00457-0	2	human: faeces	2021	+	–
22-SA00394-0	3	food:meat-based chicken products	2021	+	–
22-SA00395-0	3	food:meat-based chicken products	2021	+	+
22-SA00397-0	4	environment: livestock	2018	+	+
22-SA00398-0	4	environment: livestock	2018	+	+
22-SA00399-0	4	environment: livestock	2020	+	–
22-SA00400-0	4	environment: food processing laboratory	2017	+	+
22-SA00401-0	4	environment: slaughterhouse	2016	+	+
22-SA00403-0	4	animal: broiler	2020	+	–
22-SA00406-0	5	animal: broiler livestock	2020	+	+
22-SA00454-0	5	human: faeces	2021	+	+
22-SA00404-0	6	animal: broiler	2020	+	+
22-SA00407-0	6	animal: broiler	2020	+	+
22-SA00408-0	6	animal: broiler	2020	+	+
22-SA00409-0	6	animal: broiler	2020	+	+
22-SA00456-0	6	human: faeces	2021	+	+

primer sequences proposed by Franco et al. for the amplification of a *sog*S-specific fragment (Franco et al., 2015a). Results of this analysis showed 100 % identity in the primer region without any mismatch, positively confirming the presence of the gene.

The pESI backbone target was not found in the two strains carrying $bla_{CTX-M-65}$, as described previously for the lineage circulating in the U.S. (McMillan et al., 2020).

Notably, the two U.S. strains harboring pESI-like plasmids carrying the $bla_{CTX-M-65}$ showed the presence of more ARGs, in particular *aac*(3)-IVa, *aph*(4), *floR* and *fosA3*, in contrast to the pESI-like $bla_{CTX-M-1}$ mainly isolated in the EU (Alba et al., 2021; Roca et al., 2015).

It was noticed that only three strains carried bla_{TEM-1}, which is a non-ESBL variant gene, and two of them harbored an additional plasmid with IncX replicon, hinting towards the location of the bla_{TEM-1} gene on this plasmid, as previously described (Di Marcantonio et al., 2022).

In line with findings previously reported by Alba et al. on other strains, carbapenemase encoding genes were not observed in our study (Alba et al., 2020a).

Additionally, numerous virulence factors were identified. Ybt system and fimbriae coding genes, involved in the enhancement of *Salmonella* survival in adverse conditions and in the increase of the adhesion in human and animal cells, were found in the majority of strains. The Ybt operon, involved in iron acquisition, increases the ability of *Salmonella* to survive in low iron conditions (McMillan et al., 2020; Foley et al., 2008; Kang et al., 2006).

It was noticed that the only strain without the plasmid lacked the virulence genes *fae, fyu, irp* and *ybt* operon (Di Marcantonio et al., 2022; García-Soto et al., 2020), thus validating the association of certain

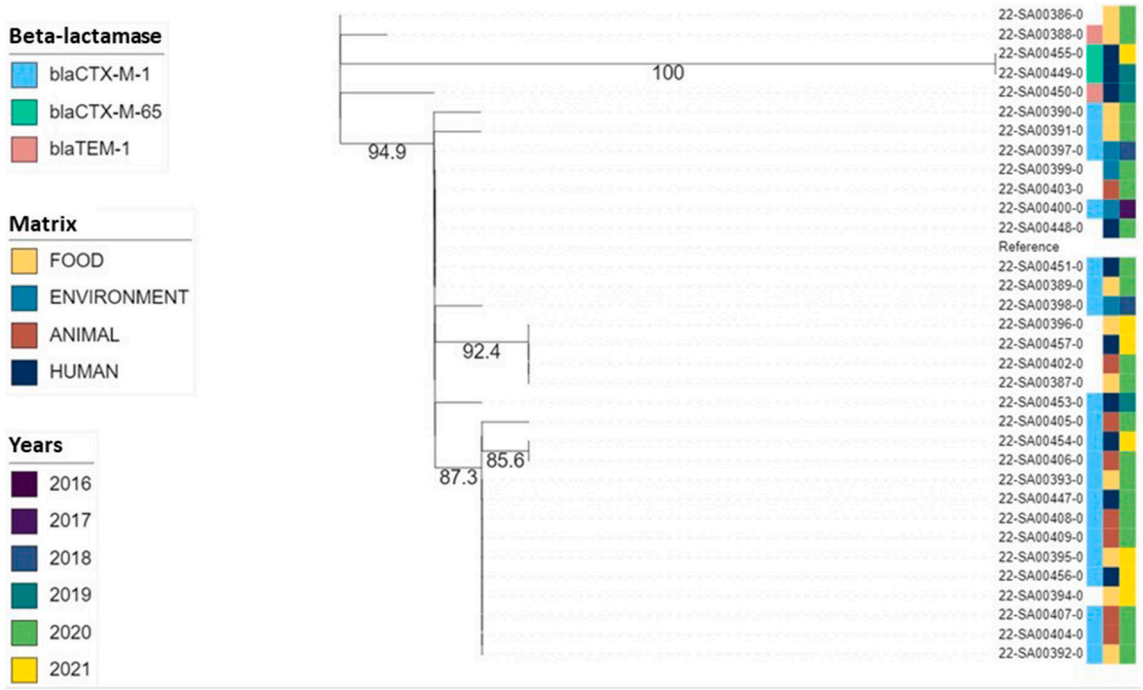

Fig. 2. SNP-based phylogenetic tree based on the WGS of pESI-like plasmid. The plasmids are colored according to strain isolation source, ESBL content and year of isolation. The tree shows the phylogenetical correlation between pESI-like plasmid isolated in *S.* Infantis strains used for this study. All plasmids can be included in the same cluster since the maximum SNP distance matrix of 18.

virulence genes with the pESI-like plasmids. Indeed, the long read sequencing confirmed the location of *fae*D, *fae*E, *fyu*A, *irp*1, *irp*2, *ybt*A, *ybt*E, *ybt*P, *ybt*Q, *ybt*S, *ybt*T, *ybt*U, *ybt*X virulence genes on the plasmid.

In this study, the cgMLST analysis revealed that most of the isolates belonged to one cluster (cgMLST Cluster 1) that included strains isolated from different sources along the food chain, all of them harboring the pESI-like plasmid, but not all strains carried $bla_{\text{CTX-M-1}}$ gene. To study the phylogenetical relationship between our strains in more detail and to increase the resolving power of WGS typing, SNP analysis was carried out. The results verified the cgMLST analyses and showed that the majority of strains grouped together in one cluster S1 with a minimum of one and a maximum of 41 SNPs, regardless of their isolation source. Moreover, taking a threshold of 23 SNPs, similar to that proposed by Pightling et al. in their study (Pightling et al., 2018), we assume that the isolates from this study evolved from a common ancestor. When using a stricter threshold of 10 SNPs, we observed six subclusters with isolates from different origins. Since our strains were isolated in a restricted geographic area, we assume a clonal spread of these pESI- like plasmids harboring *S.* Infantis strains along the food chain (Di Marcantonio et al., 2022; McMillan et al., 2020) reflected by their close genetic relationship. This is supported by the presence of other *S.* Infantis isolates in the outgroup of cluster S1, which also harbor the pESI like plasmid, but show a genetic distance of a maximum of 314 SNPs in phylogenetic analysis. For those isolates we assume a varying origin and not the same ancestor as for the cluster S1 isolates.

Plasmids can acquire genes, virulence factors and mobile genetic elements and could evolve under different selective pressure than the chromosome (Ilhan et al., 2019). For this reason, presence of plasmid in the cell can influence SNP analysis and could results in a misinterpretation of the resulting clusters (Li et al., 2019). For further insight, we estimated two maximum-likelihood phylogenetic trees – one based on core SNPs in the bacterial chromosome, the other based on core SNPs in the plasmid sequence. Finally, plasmid-based SNP phylogeny was carried out and compared with *S.* Infantis chromosomes to verify whether the plasmid co-evolves with the host chromosomes, or an independent evolution might hint towards several independent plasmid uptakes into the *S.* Infantis population analyzed in this study. Compared to chromosome based phylogenetic analysis, plasmid SNP analysis showed a great similarity within all strains harboring pESI-like megaplasmid. The presence of three type II addiction (toxin–antitoxin) systems, found in the pESI-like plasmids, seems to contribute to the stability of plasmids during vertical transmission (Lee et al., 2021).

According to Proietti et al. (Proietti et al., 2020), our results suggest that the spread might have occurred, in particular, in Italian poultry production chain, since genetically highly related strains were isolated from different points along the meat production line. However, results of WGS analysis are not sufficient to infer the direction of the transmission.

Food pathogens can undergo evolutionary changes in the path they take from farm to fork, since they acquire or loose genes or undergo other genetic mutations, especially over long-time scales. The results of this study underline the importance of phylogenetic studies to monitor and understand pathogens spread in a One Health approach.

Although WGS is now a routine procedure, supporting epidemiologic investigations and surveillance of foodborne pathogens, short-read sequencing technology faces limits such as resolving repetitive regions, which introduce ambiguities that lead to inaccurate sequence reconstruction and incomplete and fragmented *de novo* assemblies. These gaps can lead to the inability to determine accurate genome or plasmid organization or architecture, which are important in determining if genes are co-regulated or co-transmissible in the case of genes associated with mobile elements. Even though the short-reads are accurate, closed whole genome assemblies are now commonly accomplished using a combination of both short-read (for base accuracy) and long-read sequencing technologies. Indeed, it is difficult to determine the exact location of AMR genes using short read WGS-based approaches alone. Moreover, this information is important to determine risk assessment of AMR transmission. The use of long read sequencing such as Oxford Nanopore Technology (ONT) would be of added value in this context since it allows to overcome this limit (Berbers et al., 2020).

5. Conclusions

Our study reported the occurrence of *S.* Infantis strains harboring pESI-like plasmids, carrying $bla_{CTX\text{-}M\text{-}1}$ genes conferring resistance to 3rd generation cephalosporins, at different sampling points along the food chain in Central Italy. Close genetic relationship of the $bla_{CTX\text{-}M\text{-}1}$ carrying *S.* Infantis isolates harboring a pESI-like IncFIB(K)_1_Kpn3 megaplasmid confirmed the spread of a *S.* Infantis pESI-like $bla_{CTX\text{-}M\text{-}1}$ clone in samples from farm to fork.

The genetic homogeneity reported in *S.* Infantis pESI-like positive isolates of our study suggests that, although the *S.* Infantis population is heterogeneous in Europe, pESI-like variants are genetically similar each other. Those plasmids, once acquired, provide the strains a selective advantage that enhance their colonization ability and increases fitness, thus enhancing their spread (Aviv et al., 2014; Alba et al., 2020a; Carfora et al., 2018).

In line with the WHO One Health approach (Di Marcantonio et al., 2022; Alba et al., 2020a), the control of the spread of *S.* Infantis pESI-like is essential, since this clone can be resistant to 3rd generation cephalosporin considered CIAs for human health. The misuse of antibiotics or the extensive use of disinfectants, such as quaternary ammonium compounds or heavy metal used in agriculture, could increase the selection of resistant foodborne pathogens. For this reason, the monitoring of antibiotic use in livestock and the environment, and the studies on mobile genetic elements involved in AMR transmission are necessary to fight against the antimicrobial resistance threat.

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CRediT authorship contribution statement

JF and GA conceived the original idea. IR, JF and LU planned the experiments and wrote the manuscript. MN provided strains used in the study. IR conducted the experiments. GA and GB took care of funding acquisition, supervision, and project administration. GB, GFS and FA took charge of data analysis and writing – review and editing.

Declaration of competing interest

None.

Data availability

Sequencing data for isolates of this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the Bioproject accession number PRJNA1028803.

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