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Genomic characterization of multidrug-resistant monophasic *Salmonella* 4,[5],12:I:- ST34 circulating along the food chain of Central Italy

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ABSTRACT

The monophasic variant 4,[5],12:i:- of *Salmonella enterica* serovar Typhimurium (MVST) has emerged as a significant global public health concern. This study employs whole-genome sequencing (WGS) to characterize MVST isolates from Central Italy, focusing on antimicrobial resistance (AMR) genes, plasmids, virulence factors, the variability in the former *fljBA* operon region, and the genetic diversity of isolates through hierarchical cluster analysis to assess potential transmission pathways of circulating MVST clones in the food chain.

Multidrug-resistant (MDR) MVST isolates ($n = 28$) from multiple sources (2015–2021) were characterized by real-time PCR and WGS (Illumina and Oxford Nanopore Technologies).

Phenotypic and genotypic resistance profiles revealed the presence of different ASSuT profile variants (ASSuT, ASSuT-plus -additional resistances beyond the classical ASSuT profile-, and incomplete-ASSuT). Twenty-nine resistance genes across eleven antimicrobial classes were identified. Genes responsible for the classical ASSuT phenotype were highly prevalent but often complemented by further, mainly plasmid-borne, resistance genes. In-depth analysis of the former *fljBA* operon region revealed an extensive genetic variability, including the presence of ASSuT resistance genes (bla_{TEM-1}, *strA/aph(3″)-Ib, strB/aph(6)-Id*, *sul2,* and *tet*(B)), heavy metal resistance genes (HMRGs; *arsR* and *mer* operon genes), and transposon insertions (IS*10L*, IS*26*, and IS*1R*). cgMLST analysis revealed the presence of two main clusters and suggested a potential transmission of this pathogen along the food chain.

Global cgMLST analysis revealed that cluster II isolates presented an Italian endemic sub-lineage, whereas cluster I isolates were aligned broadly with international isolates, indicating their characteristic dissemination and persistence in the global food chain. These results emphasize the need for integrated genomic surveillance along with One Health monitoring.

1. Introduction

Salmonella is among the most important zoonotic foodborne pathogens; 93.8 million cases of gastroenteritis were reported annually worldwide, resulting in about 155,000 deaths (Galán-Relaño et al., 2023). In the European Union (EU), the last zoonoses report listed salmonellosis caused by non-typhoidal *Salmonella* as the second most reported gastrointestinal infection (77,486 human cases), with *Salmonella Enteritidis* and *Salmonella Typhimurium* being the serovars of highest importance among those contributing to human infections (EFSA and ECDC, 2024). The widespread dissemination of antimicrobial-resistant *Salmonella* strains along the food chain is a major threat to public health (Baker et al., 2018; Kumar et al., 2025; Mastrorilli et al., 2018).

Over the past three decades, the emergence of a monophasic variant of *Salmonella enterica* serovar Typhimurium (MVST), designated as 4,[5],12:i:-, both in human infections and animals, has been observed (Clark et al., 2020; Crayford et al., 2014; Sun et al., 2020). MVST was first identified in Portugal in the 1980s in poultry (Machado & Bernardo, 1990). Since then, it has rapidly evolved, causing outbreaks in several countries, including Italy, where it has been primarily spread through

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pork products (Di Giacomo et al., 2024; Lettini et al., 2014; Napoleoni et al., 2023; Russini et al., 2022). Further outbreaks have been reported in Luxembourg through pork meat (Mossong et al., 2007), the USA through water and pork (Kawakami et al., 2016; Kozlica et al., 2010), and Spain through pork sausage (Arnedo-Pena et al., 2016). A multi-county outbreak of MVST ST34 linked to Belgian chocolate products was investigated recently (Lund et al., 2022).

MVST is mostly isolated from pigs and accounts for 27.9% of all *Salmonella* isolates from pigs in the EU, as reported in the One Health Zoonoses report 2024. Lower frequencies of MVST are found in cattle (2.0%), turkeys (1.8%), laying hens (1.5%), and broilers (0.98%), while it is rarely detected in other animal sources (EFSA and ECDC, 2025; Marin et al., 2020). In 2023, a total of 5136 human cases of MVST were reported in the EU/EEA, making it the third most frequently isolated serotype from human cases that year (EFSA and ECDC, 2025). In contrast, since 2011, MVST has emerged as the most commonly isolated serovar from human cases in Italy, likely due to its unique ecological and epidemiological adaptations (Napoleoni et al., 2023).

In general, MVST has multiple clonal lineages that emerged in different geographical areas (Soyer et al., 2009). The lack of expression of the second phase can be attributed to multiple factors, including deletions (partial or complete) in the *fljBA* operon. Additionally, mutations in the *hin* and *fljA* genes, as well as alterations in the promoter regions regulating *fljB* and *fliC* gene expression, which are involved in phase variation, can also contribute to the loss of antigen expression (Sun et al., 2020).

So-called European, Spanish, Southern European, and U.S./American clones are important genetic lineages representing the monophasic variant of *S. typhimurium* (Moreno Switt et al., 2009; Vázquez et al., 2024).

The European clone, belonging to sequence type (ST) 34, is widely studied: It was first described in Luxembourg in 2005 (Mossong et al., 2007), and has since spread in several other countries, including in Europe, Asia (China, Japan), and the United States (Arai et al., 2018; Luo et al., 2023).

The European clone's epidemiological success is largely driven by its acquisition of specific antimicrobial and heavy metal resistance genes (Petrovska et al., 2016; Sun et al., 2020). MVST ST34 isolates belong to phage type DT193 and DT120, and have shown several variations in the deleted regions extending from *hin* to *fljA, fljB,* and STM2763-STM2745 (Arai et al., 2021; Lucarelli et al., 2010). This clone is recognized by the chromosomally encoded (R-ASSuT) resistance type (ampicillin, streptomycin, sulfonamides, and tetracycline, conferred by genes $bla_{\mathrm{TEM-1}}$, *strA/aph(3″)-Ib* and *strB/ aph(6)-Id*, *sul2*, and *tet(B)*, located in certain resistance regions (RR1-RR2/RR3), typically surrounded by IS*26* elements (García et al., 2016; Lucarelli et al., 2012; Mandilara et al., 2021; Tassinari et al., 2019).

RR1 is involved in conferring resistance to streptomycin, ampicillin, and sulfonamides and occurs in combination with RR2, conferring resistance to tetracycline. Alternatively, RR3 encodes all the above-mentioned resistances and is also flanked by IS*26* sequences. All three described resistance regions are associated with the ASSuT MVST ST34 phenotype; their evolutionary connection is still not fully understood (García et al., 2016; Sun et al., 2020; Wang et al., 2023). The integration of these RRs into the *fljBA* operon and its neighboring region disrupted the normal function of this operon, and is responsible for the ASSuT profile, which likely contributed to the selection and spread of the European clone (ASSuT phenotype, RR1-RR2/RR3 resistance regions, ST34) in pig breeding facilities, where the use of antibiotics has exerted strong selective pressure (Cadel-Six et al., 2021).

This is consistent with findings from EU member states, where high resistance rates to ampicillin (86.0%), sulfonamides (92.6%), and tetracycline (78.1%) have been reported in MVST isolates (EFSA and ECDC, 2025). This resistance profile, along with streptomycin, is considered the most characteristic feature of the "European clone" (Hopkins et al., 2010).

In contrast, all other MVST clones (Spanish, Southern European, and U.S./American) belong to ST19. Whereas the US clone is susceptible to antibiotics (Agasan et al., 2002; Soyer et al., 2009), Spanish and South European clones are multidrug-resistant (Arrieta-Gisasola et al., 2020; Vázquez et al., 2021), but their resistances are plasmid-borne, mainly involving the plasmid incompatibility groups IncC and IncR (Vázquez et al., 2021) which were also reported to harbor pSLT-derived virulence genes (García et al., 2014).

Despite its growing epidemiological significance in Italy, the genomic epidemiology of MVST strains circulating in the country remains poorly understood. To address this knowledge gap, this study aimed to characterize MVST isolated from the food chain in the Marche region of Central Italy using whole-genome sequencing (WGS). Our primary objectives were to define the genomic landscape of MVST, with a focus on antimicrobial resistance (AMR) genes, plasmid content, and virulence factors that contribute to its pathogenicity. Additionally, we investigated the genetic diversity in the *fljBA* genetic region and explored possible transmission pathways of this serovar. The results of this study will provide valuable insights into the genomic epidemiology and potential public health implications of MVST in Central Italy.

2. Materials and methods

2.1. 'Farm-to-fork' Salmonella strain panel

Salmonella spp. strains (n = 28, Table 1) investigated in this study were selected from the MVST isolates obtained during routine surveillance by the Regional Reference Centre for Pathogenic Enterobacteria (CRRPE) of the Marche Region (Central Italy) at the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (IZSUM), Peripheral Health Structure of Tolentino, Italy, between 2015 and 2021. The subset was purposively assembled to cover the farm-to-fork continuum and to include isolates from different years and source categories (food, food-related environments, animals, and human clinical samples), thereby maximizing source and temporal heterogeneity within the collection. We use the term "subset" to indicate coverage of the main matrices sampled by the regional surveillance system; however, this collection was not designed as a formal statistically representative sample of all MVST circulating in the Marche Region, and therefore, it should not be used to infer population prevalence. These 28 MDR MVST strains were isolated at different stages of the food chain: nine strains from food samples (six from pork meat, one from a bivalve mollusk, one from bovine meat, and one from mixed pork-turkey meat); three from swabs of food-related environments (two from slaughterhouses and one from a food processing laboratory); six from animals (three from swine, one from fattening pig, one from laying hen and one from turkey) and ten from human clinical samples (nine from faeces and one from blood), see also table S1.

Salmonella spp. isolation was conducted in the framework of the official controls established by the Regulation (EC) No 2073/2005 on microbiological food safety criteria (European Union, 2005), the National Poultry monitoring plan (UNI EN ISO 6579-1:2017/Amd 1:2020; *ISO 6579-1:2017/Amd 1:2020; Microbiology of the Food Chain—Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella—Part 1: Detection of Salmonella spp.—Amendment 1: Broader Range of Incubation Temperatures, Amendment to the Status of Annex D, and Correction of the Composition of MSRV and SC,* 2020; Italian Ministry of Health, 2013, 2016, 2019) and during self-monitoring controls both in food (European Union, 2005) and veterinary sectors (Italian Ministry of Health, 2013, 2016, 2019). Human clinical strains were isolated from patients with gastrointestinal diseases, some hospitalized, and were collected at the CRRPE from hospital laboratories participating in the Enter-Net surveillance network for the Marche Region.

Table 1
Strain overview showing isolation source (category and sample matrix), year of isolation, phenotypic and genotypic ASSuT type, and cgMLST-cluster information of the 28 MVST investigated strains.

Strain ID	Isolation source	year of isolation	Sample matrix	Phenotypic R-Type (pR-type)	Genotypic R-Type (gR-type)	cgMLST-cluster
22-SA00418–0	FOOD	2020	processed meat product (pork)	ASSuT	ASSuT	I
22-SA00419–0	FOOD	2020	processed meat product (pork)	ASSuT	ASSuT	I
22-SA00420–0	FOOD	2020	pork-turkey mix	ASSuT	ASSuT	II
22-SA00421–0	FOOD	2020	processed meat product (pork)	ASSuT	ASSuT	outlier
22-SA00422–0	FOOD	2020	pork meat	ASSuT	ASSuT	II
22-SA00423–0	FOOD	2020	processed meat product (pork)	ASSuT	ASSuT	II
22-SA00424–0	FOOD	2020	bovine meat	ASSuT	ASSuT	II
22-SA00425–0	FOOD	2020	pork meat	ASSuT	ASSuT-plus	I
22-SA00426–0	FOOD	2021	fishery product mollusks	ASSuT	ASSuT-plus	I
22-SA00427–0	ENVIRONMENT	2018	food processing laboratory swab	ASSuT	ASSuT-plus	II
22-SA00428–0	ENVIRONMENT	2015	swine surface swab	ASSuT-plus	ASSuT	II
22-SA00429–0	ENVIRONMENT	2015	swine surface swab	ASSuT-plus	ASSuT	I
22-SA00430–0	ANIMAL	2018	swine faeces	ASSuT-plus	ASSuT	I
22-SA00431–0	ANIMAL	2018	fattening pig faeces	ASSuT-plus	ASSuT-plus	II
22-SA00432–0	ANIMAL	2019	turkey overshoe	ASSuT-plus	ASSuT-plus	I
22-SA00433–0	ANIMAL	2017	swine organ	ASSuT-plus	ASSuT-plus	II
22-SA00434–0	ANIMAL	2017	laying hen organ	ASSuT-plus	ASSuT-plus	II
22-SA00435–0	ANIMAL	2016	swine faeces	ASSuT-plus	ASSuT-plus	II
22-SA00468–0	HUMAN	2020	faeces	ASSuT-plus	ASSuT-plus	I
22-SA00469–0	HUMAN	2020	faeces	ASSuT-plus	ASSuT-plus	II
22-SA00470–0	HUMAN	2020	faeces	ASSuT-plus	ASSuT-plus	II
22-SA00471–0	HUMAN	2020	blood	ASSuT-plus	ASSuT-plus	II
22-SA00472–0	HUMAN	2020	faeces	ASSuT-plus	ASSuT-plus	outlier
22-SA00473–0	HUMAN	2020	faeces	ASSuT-plus	Incomplete-ASSuT	I
22-SA00474–0	HUMAN	2020	faeces	Incomplete-ASSuT	Incomplete-ASSuT	II
22-SA00475–0	HUMAN	2020	faeces	Incomplete-ASSuT	Incomplete-ASSuT	I
22-SA00476–0	HUMAN	2021	faeces	Incomplete-ASSuT	Incomplete-ASSuT	II
22-SA00477–0	HUMAN	2021	faeces	Not determined	ASSuT	II

2.2. Molecular confirmation at the genus level

2.2.1. DNA extraction via thermal lysis

Single colonies from overnight cultures on Tryptic soy agar (TSA, Biolife) plates were resuspended in 300 µL of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) and heated to 100 °C for 10 min. The suspensions were then centrifuged at 14,000 rpm for 2 min, supernatants were diluted 1:3 with TE buffer, and each DNA sample was stored at −20 °C and used for all amplification assays described below.

2.2.2. Salmonella-specific real-time PCR

Strain identity at the genus level was confirmed by real-time PCR using a previously published protocol (Amagliani et al., 2010). Briefly, a 155 bp sequence of the *Salmonella* genus-specific *ttr*C gene was amplified by using *ttr1* (GCAGGAGGTCTGAATGACG) and *ttr2* (TTTTCCGCCAGTGAAGATAAC) primers (0.6 µM each) and P25 (ACT-CATCATTGAAGAAGTGCTGGCTCAC) 5' FAM labelled probe (0.1 µM). The thermal protocol included a denaturation step at 95 °C for 10 min; 30 cycles at 95 °C for 20 s and 63 °C for 1 min (Russo et al., 2024). Real-time PCR was performed in 25 µL with Premix Ex Taq (Probe qPCR) (TaKaRa) and 5 µL of DNA. *S. enterica* subsp. *enterica* ATCC 13076 was used as a positive control.

2.3. Serotyping

Serotyping of Salmonella strains was performed according to ISO/TR 6579–3:2014 (ISO/TR 6579–3:2014). *Antigenic formula and serovar were determined using the White Kauffman Le Minor scheme* (Grimont & Weill, 2007) *by using slide agglutination of each strain with Salmonella antisera (Statens Serum Institut, Copenhagen, Denmark). Phase inversion was induced to determine the second phase by pouring the Sven Gard medium (previously dissolved in a boiling water bath) into a Petri dish, waiting for it to solidify, and then placing 3–4 drops of flagellar serum from the already defined phase at the center of the dish and inoculating it with a loop of culture. After incubation at 37 °C for 24 h, the culture was tested with flagellar sera to determine whether the other flagellar phase was present.*

2.4. PCR-based confirmation of the monophasic variant of S. typhimurium

The multiplex real-time PCR assay was performed for the molecular confirmation of MVST by using three targets: *fliA-IS200* for *S. typhimurium* confirmation and its differentiation from other non-Typhimurium serovars with antigenic formula 1,4,[5],12:i: (expected to be present in all our strains), and two specific DNA targets of genes associated with second H phase flagellar antigen expression (*fljB-hin* and *hin-iroB*). The *fljB-hin* and *hin-iroB* intergenic region targets, which are expected to be present in biphasic *S. typhimurium*, were utilized to confirm the absence of corresponding DNA regions in the monophasic variants (Maurischat et al., 2015).

2.5. Antibiotic susceptibility testing (AST)

The AMR profiles of strains investigated in this study were determined by the disk diffusion method (CLSI, 2022) towards a wide panel of antibiotic agents, including critically important antimicrobials provided by the Enter-Net network coordinated by the Italian National Institute of Health (ISS) (Volkov, 2024): ampicillin (AMP, 10 μg), amoxicillin + clavulanic acid (AMC, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), meropenem (MEM, 10 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NA, 30 μg), pefloxacin (PEF, 5 μg), gentamicin (CN, 10 μg), streptomycin (S, 10 μg) and highly important antimicrobials (Volkov, 2024): cefoxitin (FOX, 30 μg), tetracycline (TE, 30 μg), chloramphenicol (C, 30 μg), trimethoprim-sulfamethoxazole (SXT, 23.75/1.25 μg), sulfisoxazole (ST, 300 μg), trimethoprim (TMP, 5 μg). *Escherichia coli* ATCC 25922 was used as a control strain.

2.6. Pulsed-field gel electrophoresis-S1 (PFGE-S1) plasmids size characterization

Pulsed-field gel electrophoresis (PFGE) was performed for plasmid size confirmation according to the PulseNet protocol, utilizing the CHEF-DR III system (Bio-Rad Laboratories GmbH, Munich, Germany). The DNA embedded in agarose was digested with the S1 nuclease. The PulseNet reference strain *S. enterica* serovar Braenderup H9812 was digested with the restriction endonuclease *Xba*I (Thermo Fisher Scientific, Darmstadt, Germany) to serve as a size standard, allowing for precise band pattern identification. Electrophoresis conditions for S1 cut blocks were: 1 s–25 s, 17 h (run time), 6 V/cm (200 V) and 120 V (Rodríguez et al., 2009).

2.7. Whole genome sequencing (WGS)

Overnight cultures in Luria-Bertani (LB) broth were used for DNA extraction with the PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). Short-read sequencing libraries were then prepared using the Nextera™DNA Flex Library Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's protocol. Paired-end sequencing was performed in 2 × 149 cycles on the Illumina NextSeq™ 500 benchtop sequencer using the NextSeq™ 500/550 Mid Output Kit v2.5 (300-cycle) (Illumina).

Moreover, long-read sequencing by Oxford Nanopore Technologies (ONT) was performed for all MVST strains. ONT libraries were prepared using the Rapid Barcoding Kit 96 V14 (SQK-RBK114.96, Oxford Nanopore Technologies, Oxford, UK). Libraries were sequenced on a MinIon Mk1C device using a FLO-MIN114 R10.4.1 flow cell. Obtained reads were filtered using nanofilt (v2.8.0) (De Coster et al., 2018).

For assembly, either the hybrid assembly software unicycler (v0.4.8) (Wick et al., 2017a; Wick et al., 2017b), which takes long- and short-reads as input, or the long-read assembly software flye (v2.9) (Kolmogorov et al., 2019) was used. Flye assemblies were subsequently polished with Illumina data using pilon (five iterations) (Walker et al., 2014)

2.8. Bioinformatic analysis

2.8.1. Genotypic characterization and hierarchical cluster analyses

Genome assemblies were subjected to the BakCharak pipeline v3.1.6 for bacterial characterization (https://gitlab.com/bfr_bioinformatics/bakcharak). The BakCharak pipeline includes different modules as follows: for AMR gene detection (tool: AMRfinder v3.12.8, database: ncbi-amrfinder (2024-01-31.1) (Feldgarden et al., 2019)), for plasmid marker detection (tool: abricate v1.0.1 (https://github.com/tseemann/abricate), database: CGE plasmidfinder (2021-03-27) (Carattoli et al., 2014)), for serotyping (tool: SISTR v1.1.1 (Yoshida et al., 2016)), for 7-gene MLST determination (tool: mlst v2.23.0, database: pubmlst 2023-01-15), and for assembly annotation (tool: bakta v1.9.3 (Schwengers et al., 2021)). Heat maps were generated in R (v4.2.2) to visualize the localization of acquired antimicrobial resistance genes and resistance-associated point mutations, as well as stress resistance genes in the tested MVST strains, distinguishing between chromosomal and plasmid-borne genes. Additionally, an overview of plasmid markers was also provided. To analyse the prophage sequence and the distribution of the *sspH2* gene, the PHASTEST analysis was conducted using the representative *sspH2*-positive and negative strains on the PHASTEST web server (https://phastest.ca/) (Wishart et al., 2023).

cgMLST analysis was performed using the chewiesnake pipeline (v3.2.2) (Deneke et al., 2021). The cgMLST scheme used is a modified version of the "Enterobase scheme" and contains 3000 Loci. The resulting allele distance matrix was hierarchically clustered using single-linkage clustering, and the resulting tree was visualized using the iTOL v7 (Interactive Tree of Life) online tool (Letunic & Bork, 2024).

2.8.2. Genetic variability within the fljBA operon region of MVST from this study

To analyse the genetic variability among the isolates of this study, the genetic region encompassing the *fljBA* operon region, which is associated with the monophasic phenotype, was analysed in Geneious Prime 2024.0.7. For this purpose, the genetic regions from *srlA* to *iroB* in the bakta annotated genome assemblies were closely investigated. A linear comparison of the genomes was generated using Easyfig (v2.2.5), allowing us to visualize the genetic differences between the isolates. Four isolates showing high heterogeneity in this genetic region could not be depicted due to incomparability.

2.8.3. Comparative genetic analysis of ST34 strains with sequence data obtained from NCBI

To investigate the genetic relatedness of isolates from an international perspective, a WGS-based comparison was conducted with MVST ST34 isolates from the NCBI database. For a targeted comparison, a curated international dataset comprising short-read data from five countries (the US, the UK, Vietnam, Italy, and Australia) was utilized, which had been previously analysed (Ingle et al., 2021). The short-read data was also processed using the AQUAMIS pipeline (v1.4.2) and underwent quality control according to the same criteria as those applied

to the strains in this study. Following quality control, 281 out of 309 assemblies passed our AQUAMIS quality thresholds and were subsequently characterized using the BakCharak and chewiesnake pipelines, as described in chapter 2.8.1, and used for genetic comparisons with isolates from our study.

2.9. Statistical analysis

The Fisher's exact test was performed in R (version 4.3.1) using the RStudio interface (version 2025) to assess any associations between the cgMLST clusters and genotypic R-Types, as well as the presence or absence of the *hin-iroB* intergenic region, the *sspH2* virulence gene, and plasmid contigs. A p-value <0.05 was considered statistically significant.

3. Results

3.1. MVST strain confirmation and pre-characterization

All strains ($n = 28$) tested positive in the genus-specific real-time PCR assay, confirming the genus *Salmonella*. Classical serotyping revealed that all 28 strains expressed the somatic antigen O:4 and the phase 1 flagellar antigen H:i. Following three successive phase inversion tests with H:i antisera, none of the 28 strains reacted with the phase 2 flagellar antigen H:2, suggesting their identity as MVST based on their phenotypic characteristics. Multiplex PCR analysis revealed that all 28 strains were indeed positive for the *S. typhimurium*-specific target *fliA-IS200* and negative for the *fljB-hin* intergenic target region. Moreover, 21 strains gave negative results for the *hin-iroB* intergenic target. The genetic region including the *fljB-hin* and *hin-iro*B intergenic PCR targets is involved in encoding the phase 2 flagellar antigen and the DNA invertase that is responsible for phase variation. The absence of these genes inhibits the flagellar phase variation, and these results confirm the monophasic phenotype of the tested strains. Positive results for the *hin-iroB* intergenic PCR target in seven strains indicate that parts of the *fljBA* region might be present in those isolates (Table S1).

3.2. Phenotypical AMR assessment

MDR phenotype was observed through AST, and complete resistance was found against ampicillin (AMP) (28/28, 100%), streptomycin (S) (27/27, 100%), and sulfisoxazole (ST) (27/27, 100%), followed by tetracycline (TE) (25/28, 89%). No resistance observed against meropenem (MEM) and ciprofloxacin (CIP). The lowest resistance prevalence was found against 3rd-generation cephalosporines: cefotaxime (CTX) and ceftazidime (CAZ) ($n = 1/28$, 4%) followed by 2nd generation cephalosporins: cefoxitine (FOX) (n = 2/20, 10%), nalidixic acid (NA) ($n = 3/28$, 11%), sulfamethoxazole + trimethoprim (SXT) ($n = 4/28$, 14%), and amoxicillin + clavulanic acid (AMC) ($n = 5/27$, 18.5%). The highest intermediate resistance was observed against ciprofloxacin (CIP) ($n = 6/28$, 21%), followed by nalidixic acid (NA) (n = 2/28, 7%) (Table S2).

Overall, 15 different phenotypic resistance profiles (pR-types) were detected. Among the tested strains, ten (37%) exhibited the typical MVST ST34-associated ASSuT resistance profile, which includes resistance to ampicillin (AMP), streptomycin (S), tetracycline (TE), and sulfisoxazole (ST). Further 14 strains (52%) displayed an "ASSuT-plus" profile, characterized by additional resistances beyond the classical ASSuT profile. The remaining three strains (11%) showed an "incomplete-ASSuT" profile, lacking at least one of the typical ASSuT resistances. For one strain, the ASSuT-type could not be completely determined, as it was not tested for streptomycin and sulfisoxazole (Tables 1 and S1).

3.3. WGS-based genotypic analysis

3.3.1. Plasmid content

The PlasmidFinder database revealed the presence of 18 different plasmid markers in the 28 analysed isolates, as shown in Fig. S4. Isolates showed the presence of one to seven different plasmid markers, with most of the strains harboring one or three different markers. Six isolates carried no circular plasmids. The presence of multiple plasmids was frequently associated with the ASSuT-plus genotype (12/13 ASSuT-plus strains, 92.3%).

Plasmid marker IncQ1_1 was found in all isolates investigated; however, long-read sequence data revealed no circular IncQ1_1 plasmid in those isolates, but instead a chromosomal location for an incomplete version of the IncQ1_1 marker (66.5% coverage, 100% identity). Therefore, this marker was excluded from Fig. S4.

IncFIB(AP001918) _1 and IncFIC(FII)_1 plasmid markers were found as multi-replicon plasmids ranging from 87,189 bp to 118,664 bp in size in ten isolates from food, animal, and human origin. However, only one food strain carried IncFIC(FII)_1 (93,846 bp) as a single replicon. Another multi-replicon plasmid (IncHI2_1; IncHI2A; RepA_1_pKPC-CAV1321), 290340 bp in size, was detected in one isolate of human origin.

Other plasmids, belonging to the IncY_1 (95,372 bp), IncA/C2_1 (155,454 bp), and IncI1_1_Alpha (83,290 bp) family, were detected in three food-origin isolates (each carried one of them); and IncX1_1 (44,989 bp) was detected in one animal-derived isolate. Several Col plasmid markers have been detected (ColRNAI_1, Col(VCM04)_1, Col156_1, Col(MGD2)_1, ColpVC_1, and Col440I_1) that ranged less than 11 kbp in size (Fig. S4,Table S1 and Table S3).

3.3.2. ONT and PFGE plasmid size determination

ONT predicted plasmid sizes are depicted in Table S1 and correlated 100% with the S1-PFGE results, for larger plasmids such as IncF plasmids and other Inc. groups (IncY, IncX, IncA/C, IncHI2, IncI). Smaller plasmids, such as Col plasmids (<11 kbp), were not visible in PFGE gels because the plasmids ran out of the gel at the bottom due to their small size. Also, for the above-mentioned multi-replicon plasmids (that are formed by the fusion of two or more plasmids), bands of the correct size were detected in the PFGE gels (Fig. S1).

3.3.3. AMR genes and their genomic locations

AMR gene profiles obtained from hybrid assemblies identified 29 resistance genes across eleven antimicrobial classes in 28 samples. In detail, nine genes conferring resistance to aminoglycosides were observed, and among them, the most frequently identified genes were *strA/aph(3″)-Ib* and *strB/ aph(6)-Id*, found in all genomic sequences ($n = 28$). Two beta-lactamase encoding genes, $bla_{\text{TEM-1}}$ and $bla_{\text{CMY-2}}$, were found; $bla_{\text{TEM-1}}$ was found in 27 sequences, whereas $bla_{\text{CMY-2}}$ was identified only in one isolate negative for $bla_{\text{TEM-1}}$. Sulfonamide resistance genes were detected in genomic sequences as follows: *sul2* (n = 28), *sul3* ($n = 3$), and *sul1* ($n = 1$). Tetracycline resistance genes were found as follows: *tet(B)* ($n = 24$), *tet(M)* ($n = 5$), and *tet(A)* (n = 3) (Fig. S2).

The genotypic R-types (gR-types) showed the prevalence of: ASSuT 39.3% ($n = 11$), ASSuT-plus 46.4% ($n = 13$), and incomplete-ASSuT 14.3% ($n = 4$) (Table S1 and Table 1). Overall, genotypic data confirmed the MDR status in all MVST isolates, and the prevalence of key genes conferring resistance to the typical ASSuT profile accounted for the highest among all genes: *strA/aph(3″)-Ib* and *strB/ aph(6)-Id*, for streptomycin resistance, and *sul2* for sulfisoxazole resistance were detected in all isolates (100%), followed by $bla_{\text{TEM-1}}$ for ampicillin resistance (96%) and *tet(B)* for tetracycline resistance (86%).

MVST strains carried additional antimicrobial resistance genes corresponding to their observed phenotypic resistance and these genes contributed to other resistance profiles (ASSuT-plus or incomplete-ASSuT). Additional resistances were observed against chloramphenicol (C), gentamicin (CN), trimethoprim (TMP), trimethoprim-

sulfamethoxazole (SXT), quinolones/fluoroquinolones (PEF, NA). Chloramphenicol resistance genes: *floR* and *cmlA1* were found to confer resistance towards chloramphenicol (C). Gentamicin (CN) resistance was primarily conferred by *aac(3)-IId* and *aac(3)-IVa* genes. The *dfrA12* and *dfrA27* genes conferred resistance to trimethoprim, whereas the combined effect of *dfr* (*dfrA12/dfrA27*) and *sul* (*sul1/sul2/sul3)* genes contributed towards trimethoprim-sulfamethoxazole (SXT) resistance. Nalidixic acid (NA) and pefloxacin (PEF) resistance was conferred by chromosomal *gyrA* mutations (D87G, S83F) and plasmid-mediated resistance genes (*qnrB19* and *qnrS1*), which together contribute to reduced susceptibility towards quinolones/fluoroquinolones. AMR gene patterns showed inconsistencies with ASSuT and ASSuT-plus phenotypic profiles in seven cases. Three isolates: 22-SA00421–0 (food), 22-SA00427–0 (environmental), 22-SA00431–0 (animal) were ASSuT phenotypically, but they carried an ASSuT-plus genotype. In contrast, three isolates of human origin (22-SA00468–0, 22-SA00471–0, 22-SA00474–0) were phenotypically ASSuT-plus but were typical ASSuT genotypically. Strain 22-SA00422–0 was categorized as incomplete-ASSuT genotypically, although it was phenotypically ASSuT-plus. The genes conferring ASSuT phenotype were found on the chromosomal contigs in all isolates. Mostly, additional resistance genes were found on plasmids, except for *aph(4)-la*, *floR, tet(A),* and *tet(M),* which were also located on the chromosome. For example, in isolate 22-SA00475–0, *aac (3)-IVa*, *aph(4)-Ia,* and *floR* resistance genes were found on the fusion plasmid IncHI2_1; IncHI2A_; RepA_1_pKPC-CAV1321 (290,340 bp). In isolate 22-SA00422 the genes $bla_{\text{CMY-2}}$, *mph(G)*, *mef(C), tet(A),* and *floR* were found on plasmid IncA/C2_1 (155,454 bp), the fusion plasmid IncFIB(AP001918)_1; IncFIC(FII)_1 (87,189 bp) harbored the gene *aph (3′)-Ia* and the Col440I_1 plasmid (2699 bp) harbored the *qnrB19* gene. Some genes found on the chromosomes were also present in additional copies on plasmids, for example, the genes *strA/aph(3″)-Ib, strB/ aph(6)-Id*, and *sul2* were found in multiple copies on IncA/C2_1 (155,454 bp) in isolate 22-SA00422–0. Moreover, two isolates (22-SA00470–0, 22-SA00476–0) with chromosomal point mutations were found, which were associated with fluoroquinolone resistance (*gyrA_D87G, gyrA_S83F*) (Fig. S2).

3.3.4. Stress resistance genes and their genomic locations

Heavy metal resistance gene (HMRGs) count ranged from two (two isolates) to 34 (one isolate). The most frequent gene count was 20, which was found in twelve samples, followed by 24 genes detected in eleven samples. These genomic profiles represent five distinct combinations of the eight heavy metal resistance classes, thereby conferring resistance to arsenic, copper, copper/gold, copper/silver, gold, mercury, silver, and tellurium. Concerning biocides, only four MVST isolates (one isolated from pork meat and three of clinical origin) harbored genes conferring resistance to the classes of quaternary ammonium compounds. The biocide gene count was one to two (Fig. S3). No acid and heat resistance genes were found.

HMRGs were consistently found on chromosomes, except for two isolates that also exhibited the presence of HMRGs on plasmids. Among these two, one strain (22-SA00475–0) showed the presence of duplicated gene copies that were located on both the chromosome and the plasmid (fusion plasmid: IncHI2_1; IncHI2A_; RepA_1_pKPC-CAV1321 of size 290,340 bp). Additionally, only this strain carried *ter* resistance genes solely present on the same fusion plasmid. Whereas the second strain (22-SA00422–0) carried all *mer* genes on a plasmid of incompatibility group IncA/C2_1 of 155,454 bp length.

Biocide resistance genes were always found on plasmids (Fig. S3). The *qacL* gene was located on similar fusion plasmids (IncFIB (AP001918)_1, IncFIC(FII)_1) of different sizes in three isolates: 22-SA00469–0 (117,345 bp), 22-SA00472–0 (118,664 bp), and 22-SA00423–0 (110,396 bp). Whereas in the strain 22-SA00475–0, both *qacL* and *qacEdelta1* genes were located on the IncHI2_1; IncHI2A_; RepA_1_pKPC-CAV1321 fusion plasmid described above.

3.3.5. Virulence genes

Overall, 158 virulence genes were identified in our MVST isolates. Among them, 75% (n = 21/28) of the isolates carried 158 virulence genes, whereas the remaining 25% (n = 7/28) carried 157 genes. This one gene difference was based on the presence or absence of the prophage-related *sspH2* gene, which was associated with the effector delivery system. Overall, the largest number of genes was found to be related to the effector delivery system (T3SS; n = 112–113), followed by fimbrial/adherence-related genes (n = 25). Other virulence genes associated with nutritional factors (n = 7), regulation (n = 5), immune modulation (n = 2), antimicrobial activity (n = 2), stress survival (n = 1), and motility (n = 1) were found in 100% (n = 28/28) of the isolates (supplementary table S1).

The PHASTEST analysis presented that the *sspH2*-positive strain (22-SA00418–0) harbored an incomplete prophage of 14,250 bp. However, in the *sspH2*-negative strain (22-SA00419–0), a huge part of the prophage, including the *sspH2* gene (7979 bp), was lost, resulting in only fragmented phage residuals (6271 bp) Comparative genomic alignment (Easyfig) of the prophage regions between the representative strains confirmed that the *sspH2* gene region, which was present in strain 22-SA00418–0, was deleted in strain 22-SA00419–0 despite having higher sequence identity (100%) in genomic regions (Fig. S5).

3.3.5.1. Salmonella Pathogenicity Islands (SPIs) and associated key virulence genes. SPI database analysis predicted the presence of 11 distinct SPIs (SPI-1, SPI-2, SPI-3, SPI-4, SPI-5, SPI-9, SPI-11, SPI-12, SPI-13, SPI-14, SPI-16) in all MVST strains. The key virulence genes associated with SPI-1 and SPI-2, which are mainly involved in invasion, survival, and colonization of host cells, were detected in all isolates. Among them, the most important genes associated with SPI-1 type III secretion system observed were invasion and effector genes (*invA*, *invB*, *invC*, *invE*, *sipB*, *sipC*, and *sipD*) and regulatory genes (*hilA, hilC, hilD*). The core genes associated with the SPI-2 type III secretion system included the structural *ssa* genes (*ssaC*, *ssaD*, *ssaH*, *ssaJ*, *ssaK*, *ssaO*, *ssaQ*, *ssaR*, *ssaS*, *ssaT*, *ssaU, ssaV*), effector genes (*sseA*, *sseB*, *sseC*, *sseD*, *sseE*, *sseF*, *sseG*, *sseL*, *sspH2*), regulatory genes (*ssrA*, *ssrB*) and chaperon genes (***sscA, sscB***). In addition, fimbrial genes (*fimA*, *fimC*, *fi mD*, *fimF*, *fimH*, *fimI*, *fimW*, *fimY*, *fimZ*), genes involved in iron acquisition (*entA*, *entB*, *fepC*, *fepG*), and stress survival gene *rpoS* were also detected in all isolates. The combined presence of these important virulence determinants highlights the strong pathogenic potential carried by MVST isolates under study.

3.3.6. Genetic relationship of isolates: Core genome multilocus sequence typing (cgMLST) analysis

BakCharak analysis confirmed that all isolates belonged to the 7-gene MLST type 34. The cgMLST analysis revealed the presence of two main clusters and two outliers (Fig. 1). Cluster I contained ten isolates with allelic differences ranging from 8 to 34, whereas cluster II contained 16 isolates with allelic differences ranging from 650. The median allelic distance between both clusters was 46. Isolates within each cluster exhibited multiple genotypic resistance profiles (ASSuT, ASSuT-plus, and incomplete-ASSuT) and were collected from various sources during the 2015–2021 period. . In cluster I, ASSuT isolates were more frequent (n = 6), followed by incomplete-ASSuT (n = 2) and ASSuT-plus (n = 2). In cluster II, ASSuT-plus (n = 9) isolates were more frequent, followed by ASSuT (n = 5), and incomplete-ASSuT (n = 2). Both outliers were ASSuT-plus.

All *hin-iroB* PCR-positive isolates were found in cluster II (Fig. 1). The 21 isolates (six from food, three from environment, four from animals, and eight from humans) possessed 158 virulence genes, of which nine isolates belonged to cluster I, ten belonged to cluster II, and two isolates were outliers. However, of the seven isolates (three from food, two from animals, and two from humans), which harbored 157 genes and lacked the *sspH2* gene, six belonged to cluster II, and only one food isolate belonged to cluster I.

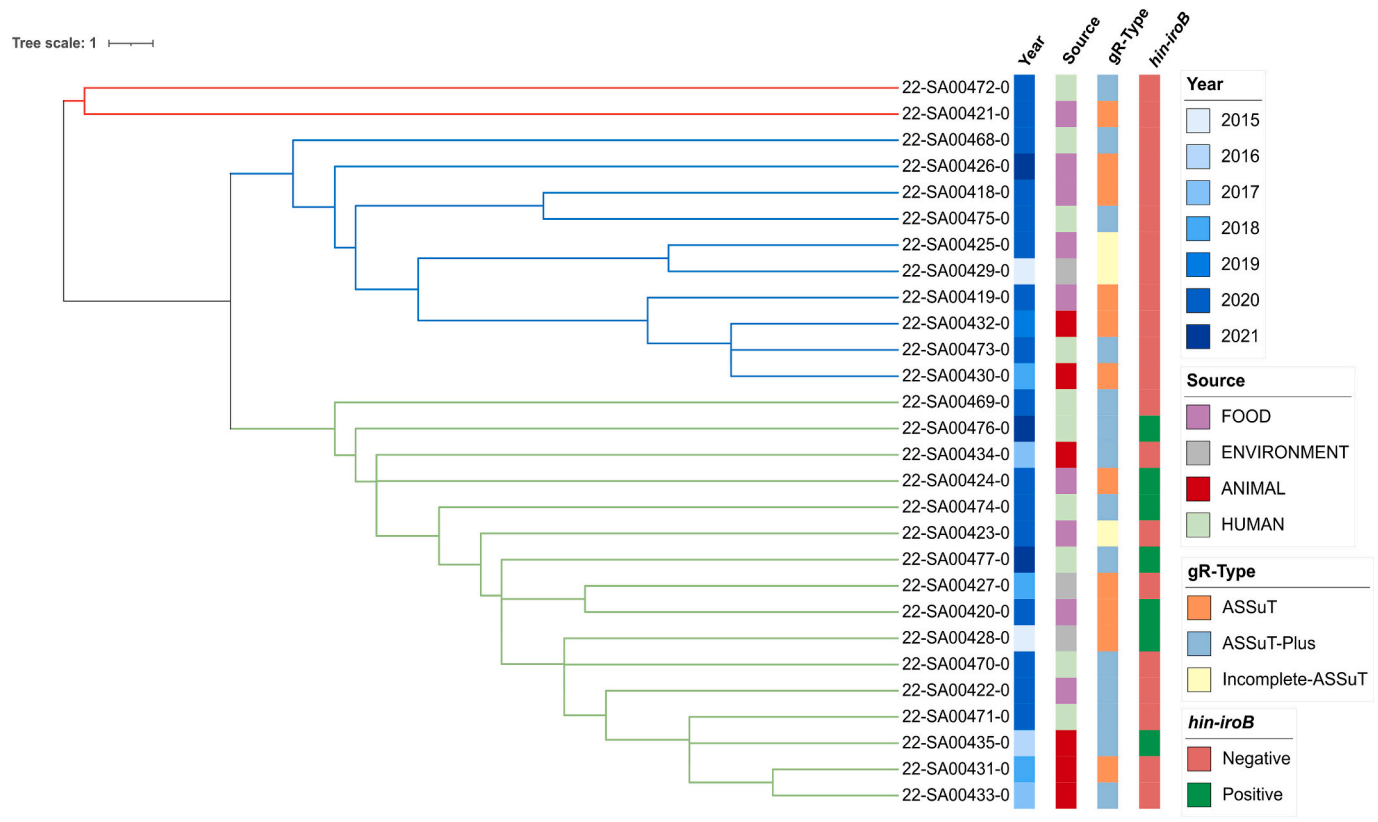

Fig. 1. cgMLST-based clustering illustrating the genetic relatedness among 28 investigated MVST strains. The first colored metadata layer indicates the year of isolation, the second indicates the isolate's source, the third shows the genotypic R-type, and the fourth corresponds to the PCR-based characterization of the *hin–iroB* intergenic region. Blue lines indicate Cluster I isolates, green lines Cluster II isolates, and red lines outliers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Among the ten isolates from cluster I, six isolates did not carry any plasmid marker, whereas the other four isolates showed two to four plasmid markers per strain. In cluster II, at least one and up to six plasmid markers per strain were found among the 16 isolates.

In detail, in cluster I, nine different plasmid markers were detected (Col(MGD2)_1; ColRNAI_1; IncFIC(FII)_1; Col156_1; ColpVC_1; IncFIB (AP001918)_1; IncHI2_1; IncHI2A_1; RepA_1_pKPC-CAV1321), whereas in cluster II, 13 different plasmid markers were found (ColRNAI_1; IncY_1; Col156_1; Col440I_1; Col440II_1; IncA/C2_1; IncFIB(AP001918) _1; IncFIC(FII)_1; IncI1_1_Alpha; Col(VCM04)_1; Col8282_1; IncX1_1; ColpVC_1).

The presence of *mer* operon genes was found to be cluster-specific, and, except for one (22-SA00425–0), all isolates of cluster I, along with an isolate (22-SA00426–0) of low biocide and metal resistance gene count (n = 6), exhibited the *mer* operon genes and predominantly showed a biocide and heavy metal resistance gene count of 24. Whereas, in cluster II, only three isolates harbored *mer* genes (mostly carrying 20 metal resistance genes). Both outliers also harbored *mer* genes with a heavy metal resistance gene count of 24.

Statistical analysis of cluster-associated markers revealed that the `hin-iroB` intergenic region was significantly associated with cluster II (p-value = 0.022), whereas cluster I isolates were all negative for *hin-iroB*. Additionally, plasmid contig counts were found to be higher in cluster II as compared to cluster I, with a statistically significant result (p-value = 0.0009121), suggesting differences in plasmid content between the clusters. No statistically significant differences ($p \geq 0.05$) were observed for genotypic R-types (ASSuT/ASSuT-plus/incomplete ASSuT), and the *sspH2* virulence gene presence/absence, although these factors showed trends across clusters. The significant associations observed between cgMLST clusters and the *`hin-iroB` intergenic region* and plasmid profiles suggest significant genetic differentiation between the two MVST clusters. Based on these results, MVST isolates in cluster II may share a common transmission chain, which is different from that of cluster I, providing insight into potential transmission pathways.

3.3.7. Genetic variability within the fljBA operon region of MVST isolates

Analysis of the extracted region from 24 selected isolates (spanning from *srlA* to *iroB*) to investigate the genetic basis for the monophasic phenotype revealed extensive genetic variability in the former *fljBA* operon region, associated with insertions of transposons (*tnp* regions), antimicrobial resistance genes (AMR), and HMRGs (Fig. 2). Transposase insertions, including IS*10L*, IS*26*, and IS*1R*, were observed. Repetitive insertions of IS*26* were detected in all tested isolates, followed by IS*1R* in 19 isolates (ten out of cluster II, eight out of cluster I, and one outlier (Fig. 2)). However, the large-sized transposase gene in IS*10L* was found in only nine isolates, out of which eight belonged to cluster I and the ninth was an outlier. The presence of IS*10L* thus appears to be also a cluster-specific characteristic of cluster I and correlates here with the presence of the *mer* operon. AMR genes: $bla_{\text{TEM-1}}$, *strA/aph(3″)-Ib, strB/aph(6)-Id, sul2,* and *tet*(B) associated with the ASSuT phenotype, along with tetracycline resistance-associated transcriptional repressors *tet*(C) and *tetR*(B), were detected in most of the extracted genetic regions under study. HMRGs encompassing the *mer* operon, harboring an array of seven genes (*merE, merD, merA, merC, merP, merT, and merR*), were found in 11 isolates, and the *arsR* gene associated with arsenic resistance was found in 20 isolates. Interestingly, *arsR* was always found in association with tetracycline-resistant genes (Fig. 2). The different results observed in multiplex real-time PCR (explained above) based on the *hin-iroB* intergenic region (positive in seven isolates of cluster II and absent in others) correlated with the variability observed in this genetic region of MVST isolates, where an incomplete *hin* gene was found in isolates

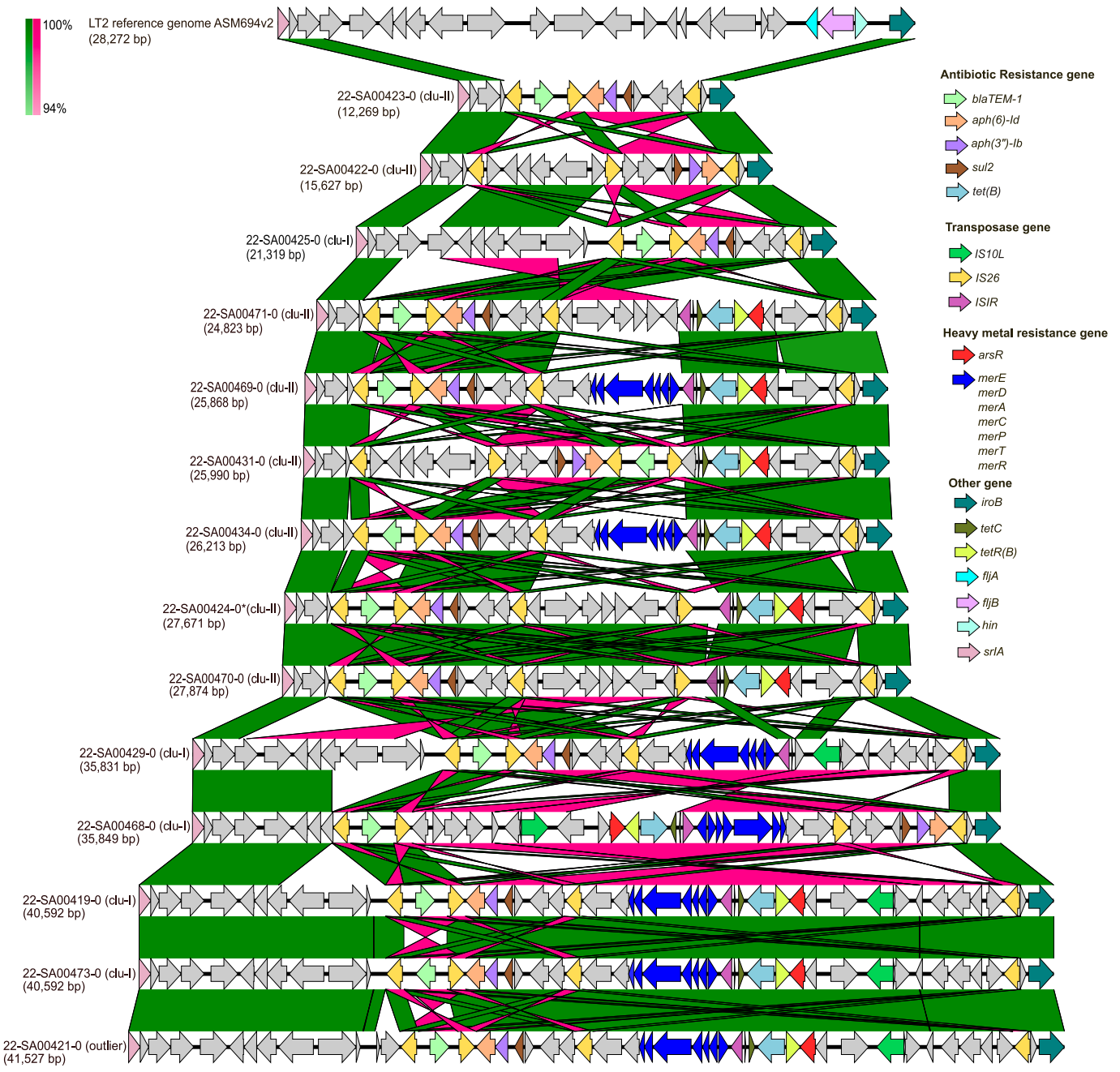

Fig. 2. Comparison of the genetic region from *srlA* to *iroB* genes associated with the monophasic phenotype shown by representative MVST isolates from a total of 24, including the reference genome *S. typhimurium* LT2 (GenBank: GCA_000006945.2). Isolates with similar genomic structures were grouped and represented by a single isolate: 471 (471 and 427), 431 (431 and 433), 424 (424, 435, 420, 428, and 477), 419 (419, 430, 475, 418, 426). The regions were sorted by size before analysis. EasyFig BLASTn was used to generate the alignment, which shows 94–100% nucleotide sequence similarity (scale at upper left). BlAST hits that connect the sequences are shown in green (direct hits) or pink (reverse hits). Gene arrows are colored according to their functions, including transposase genes, antibiotic resistance genes, and heavy metal resistance genes. If an incomplete *hin* gene was detected, resulting in a positive PCR result for the *hin–iroB* intergenic region, the isolate was marked with an asterisk (detailed data not shown). Information on isolates' cgMLST-based clustering characteristics is indicated as "clu-I", "clu-II" or "outlier" (Cluster I, Cluster II or none of the clusters, respectively), corresponding to Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with a positive *hin-iroB* intergenic region target signal (Fig. 2).

3.4. *In silico comparison with other ST34 isolates*

In the cgMLST clustering analysis, with further 281 sequences from a previous Australian study (Ingle et al., 2021). The pairwise allelic distance of isolates of the regional cluster I, with the 281 external isolates, ranged from 7 to 53, whereas for regional cluster II isolates, it ranged from 4 to 75. Thirteen isolates from our study, belonging to regional cluster II (Fig. 1), were most closely related to nine isolates from an Italian origin and four isolates from the UK (Fig. 3), although these isolates did not belong to a certain described genetic lineage in the original study (Fig. 6, (Ingle et al., 2021).

The remaining three isolates from regional cluster II (22-SA00424, 22-SA00476, 22-SA00469), which showed the largest allelic difference among cluster II isolates in our study, clustered separately and showed a

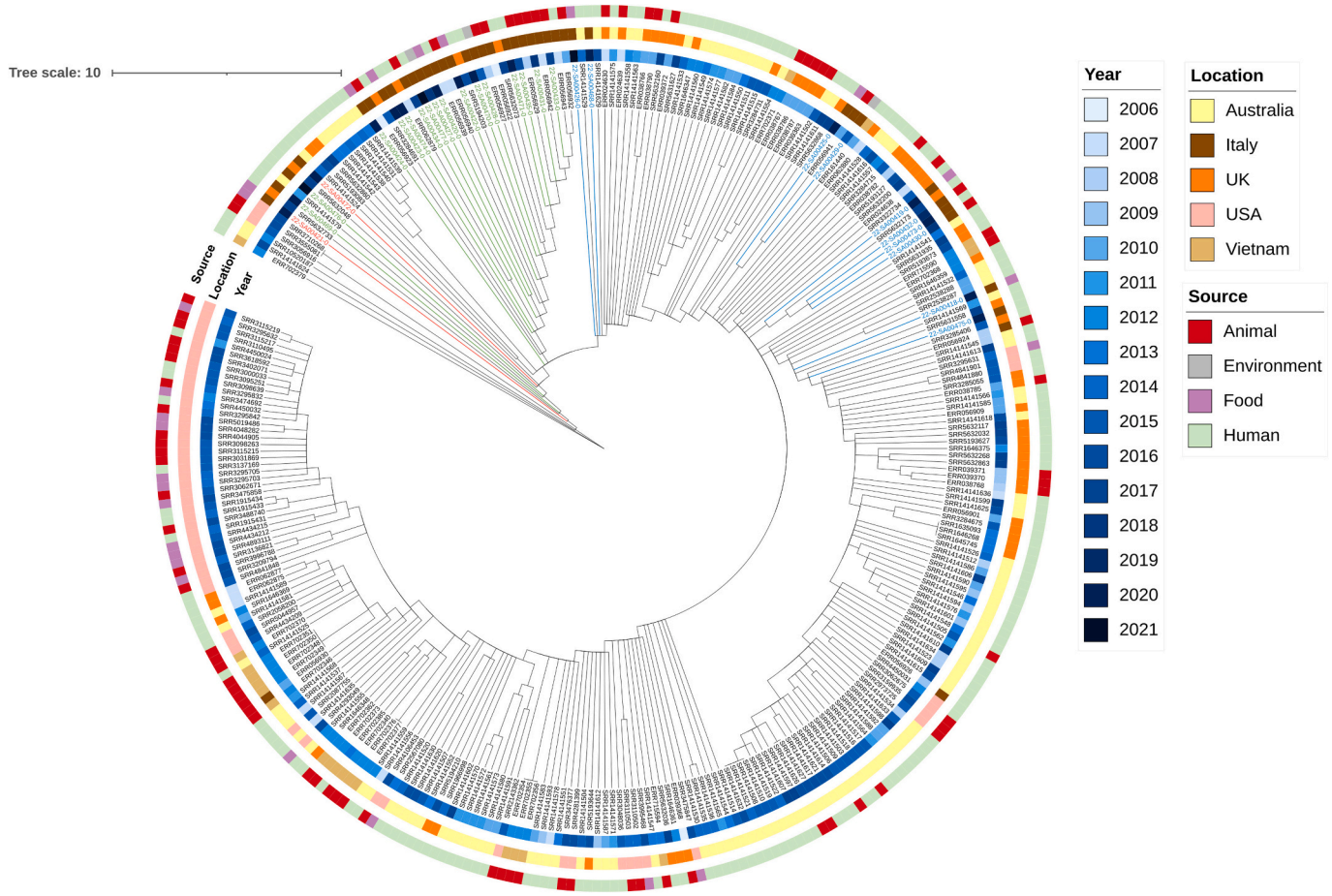

Fig. 3. cgMLST-based clustering analysis demonstrating the genetic relatedness among MVST ST34 strains (n = 309), with metadata layers showing the year of isolation, geographical origin, and isolate source. Isolates from this study are highlighted using the same colour scheme as Fig. 1: regional cluster I in blue, regional cluster II in green, and outliers in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

larger genetic distance (21–75) to the other isolates in this comparative clustering (Fig. 3).

All ten isolates belonging to regional cluster I from the current study (Fig. 1) clustered unequally among the 281 external isolates and were found dispersed within ST34 isolates from multiple countries, including Australia, Italy, the USA, Vietnam, and the UK. Outliers from our study (22-SA00472, 22-SA00421) still clustered as outliers in this comparative clustering (Fig. 3).

4. Discussion

Since 1997, a higher global incidence of the MDR MVST (ST34) clone (originally named "European clone") has imposed a notable public health concern. MVST infections have attained a status of worldwide public health alert due to their link with the swine food chain (Sun et al., 2020). As a country with a high level of pork consumption, the genomic epidemiology of MVST, along with its transmission pathways, is crucial to investigate in Italy. The current study has confirmed the circulation of the MDR MVST clone ST34 isolated from different matrices (humans, animals, food, food-related environment) in the Marche Region of Central Italy, and similar results were reported earlier (Russo et al., 2022). cgMLST-based hierarchical clustering analysis revealed the presence of two main genetic clusters, each comprising isolates from different sources across the 2015–2021 period, suggesting potential transmission along the food chain.

Our study confirmed the high prevalence of phenotypic resistance against important antimicrobials, including ampicillin, sulfonamides, streptomycin, and tetracycline, which is characteristic of MVST ST34 isolates and consistent with reports from other European member states (EFSA and ECDC, 2025). Phenotypic resistance profiles were verified on the genetic level, revealing the presence of AMR genes causing this ASSuT phenotype. However, the presence of diverse extended phenotypic AMR profiles (n = 15) indicates a higher genetic diversity, with regard to resistance mechanisms. Mostly, isolates showed an "ASSuT-plus" profile, phenotypically (52%) and genotypically (46%). The presence of additional, often plasmid-associated resistance genes in the Italian isolates may be attributed to ongoing selection pressure caused by the use of antibiotics in pig farms, as previously reported by Napoleoni et al. (2023) and in clinical settings, as evidenced by the World Health Organization (WHO, 2025). This finding is consistent with the results of our study, which showed that the majority of genotypically "ASSuT-plus" profiles were most frequently observed in human samples (66.7%) and animal samples (70%), whereas they were less common in environmental samples (33%).

The seven observed discrepancies in AMR gene patterns while comparing pR-Types and gR-Types implied that the three isolates (22-SA00421–0, 22-SA00427–0, 22-SA00431–0), which were ASSuT phenotypically but found genotypically ASSuT-plus, are explained by the presence of the kanamycin resistance gene *aph(3')-Ia*. Kanamycin, however, was not included in the phenotypic test panel. In contrast, three human isolates (22-SA00468–0, 22-SA00471–0, 22-SA00474–0) exhibited a discordant phenotype and genotype, being ASSuT-plus phenotypically, but were typical ASSuT genotypically. This discrepancy could be attributed to the loss of plasmid-borne resistance determinants during sample processing (Chung The et al., 2023) or the isolates' resistance levels that were partially close to the threshold for

susceptibility (data not shown). Strain 22-SA00422–0 was categorized as incomplete-ASSuT genotypically, which was phenotypically ASSuT-plus, because tetracycline resistance was mediated by *tet(A)* rather than *tet(B),* and ampicillin resistance was mediated by bla_{CMY-2} instead of bla_{TEM-1} in this strain, and the genes were located on a plasmid of incompatibility group IncA/C2_1 (155,454 bp).

The isolates exhibiting different gR-types were distributed across both regional clusters identified in this study; mostly "ASSuT-plus" gR-types were observed in cluster II. The frequent association of multiple plasmids expanding the ASSuT resistance profile to ASSuT-plus, likewise observed in isolates belonging to cluster II in this study, was also reported in previous studies (García et al., 2016; Ingle et al., 2021). The detection of fusion plasmids carrying multiple stress resistance genes in a human clinical isolate highlights the enhanced adaptability of plasmids to environmental stress and the potential for co-transferring resistance genes *via* mobile genetic elements (MGEs). Hence, these results are in concordance with Fang et al.'s study, which suggests the crucial monitoring of transposable elements (Fang et al., 2024). Apart from the resistance genes located on MGE, the chromosomally encoded resistance genes, characteristic of the MVST ST34 European clone, which are commonly located adjacent to the *fljBA* operon region and replace the genes responsible for second H-phase expression, were confirmed to be present in the isolates of this study. PCR-based characterization targeting the *fljB-hin and hin-iroB* intergenic regions demonstrated the absence of the *fljB-hin* intergenic region in all MVST isolates, confirming the loss of functional phase 2 flagellar antigen-encoding genes. The second intergenic PCR target (*hin-iroB*) was absent in 21 isolates, whereas seven isolates belonging to cluster II were positive for this target. Originally, we therefore assumed the presence of two different MVST lineages, based on the hypothesis that the ST34 European clone lacks the *hin* region but harbors *iroB* located upstream of the deletion region (Arai et al., 2021; García et al., 2016), and is thus supposed to be also negative for the *hin-iroB* intergenic region. However, this was not confirmed with in-depth WGS-based sequence analysis (Fig. 2), which revealed and confirmed the absence of a functional *hin* gene in all ST34 isolates. Instead, in the seven positive isolates, the presence of an incomplete *hin* gene variant was detected, which retained the *hin-iroB* intergenic region that was correctly amplified by PCR (Fig. 1 and Fig. 2). Furthermore, no monophyletic distribution of isolates showing a positive *hin-iroB* PCR result was observed, and the *fljBA* operon region showed a high degree of heterogeneity. Hence, the presence of the *hin* gene rudiments, also surrounded by transposase genes, among cluster II isolates might indicate the presence of a resistance region with an incomplete *hin* gene in a common ancestor isolate of cluster II and subsequent independent deletion or rearrangement events leading to loss of this truncated *hin* gene in some of the isolates.

Interestingly, genetic variability in the *fljBA* operon region seems to be higher in isolates of cluster II than in isolates of cluster I. This might be associated with the higher plasmid content and plasmid diversity in cluster II isolates, possibly contributing to a higher density and panel of MGE and associated resistance genes, impacting frequency of genetic rearrangements, especially in this MGE-enriched *fljBA* region. This concept has been supported in the literature from previous studies (Arrieta-Gisasola et al., 2020; Mourão et al., 2014). Presence of IS*10L* seems, however, a specific feature for cluster I isolates, which correlates with the presence of the *mer* operon, also described in García et al. (García et al., 2016).

Plasmid marker analysis revealed the presence of chromosomally integrated incomplete IncQ1 plasmid markers in all investigated MVST isolates. The presence of IncQ1 plasmid makers in WGS data from MVST was previously observed in other studies (Li et al., 2021; Petrin et al., 2023; Shi et al., 2025). Petrin et al. and Shi et al. did not investigate the completeness or position of the IncQ1 plasmid marker in detail (Petrin et al., 2023; Shi et al., 2025). Li et al., who employed long-read sequencing to improve genome assembly and resolve complex regions, found that the IncQ plasmid replicon was present in the *fliBA* chromosomal sequences of the fourteen investigated MVST isolates. They suggested that plasmid-to-chromosome recombination events transformed biphasic *S. typhimurium* to MVST (Li et al., 2021). This is in line with the theory from Lucarelli et al., who stated that by sequence comparative analysis, they found a 99% sequence identity between the RR1 and RR2 and a region of the plasmid pO111_1 from *E. coli* (Lucarelli et al., 2012; Sun et al., 2020). Plasmid marker analysis of pO111_1 in this study (data not shown) revealed the presence of four plasmid markers: IncHI1A_1, IncFIA(HI1)_1, IncHI1B(R27)_1, and an incomplete (66.5% coverage, 100% identity) IncQ1_1 marker embedded in its ASSuT resistance gene region. This is in line with findings from our study, as well as with findings from Garcia et al., hypothesizing IncH1 plasmids such as pO111_1 as the origin of RR3 (García et al., 2016; Sun et al., 2020). Hereby, recombination events within the resistance region might have taken place between the chromosomal region and homologous sequences harbored by plasmids (for example, by transposon-driven recombination, as discussed below). However, whether two independent events caused the insertion of RR1-RR2 and RR3 into the *fliBA* region, or whether the distinct regions originated from a single integration event in a common ancestor that later diverged through insertions, deletions, rearrangements, and recombination events, remains unclear. The continuous growth of genomic databases and ongoing advances in long-read sequencing are expected to further refine our understanding of the complex evolutionary processes that have shaped the emergence and diversification of MVST lineages.

In general, the variability in the genetic region responsible for the monophasic phenotype was found by multiple authors to be linked with certain transposon insertions, with IS*26* in the *fljBA* promoter region being the most common. This finding supports the hypothesis that IS*26* can identify a hotspot in the *fljBA* genomic region (Boland et al., 2015). The notion that transposon integration can create a chromosomal region prone to subsequent evolutionary changes was previously suggested by Oliveira et al. (2017). This is further supported by a study from Wang et al., which attempted to recreate the genetic mechanism involved in the generation of MVST from biphasic *S. typhimurium* through complex transposition events mediated by transposon insertions (IS*26*/IS*1R*). Therefore, they introduced plasmids carrying the resistance region 3 (RR3) into a biphasic *S. typhimurium* strain. The IS*26*-driven insertion of RR3 into an 8-bp target site located between the *hin* and *iroB* genes led to the formation of intermediate isolates, harboring both RR3 and *fliBA*. Subsequently, the replacement of the *fljBA* operon and its flanking sequences, mediated by the insertion sequences IS*26* or IS*1R*, resulted in the generation of *Salmonella* 4,[5],12:i:-. Moreover, they observed that further IS*26/IS1R*-mediated recombination events in RR3 can lead to the generation of new resistance regions, explaining the large variability we observed in our closely related isolates. Thus, they demonstrated that these mobile genetic elements are the main drivers of microevolution by increasing the genomic plasticity of *Salmonella* 4,[5],12:i:- (Wang et al., 2023).

Genotypic analysis of HMRGs revealed an abundant and diverse presence of genes responsible for heavy metal resistance against copper, gold, mercury, silver, arsenic, and tellurium across isolates belonging to all sources under study, suggesting their potential widespread distribution along the food chain in Central Italy. These results can probably be explained by the use of heavy metals as micronutrients in livestock and pig feed (Medardus et al., 2014). This genomic profile suggests that these isolates have the potential to survive in environments contaminated with heavy metals and raises public health concerns due to the potential links and co-occurrence of HMRGs and antibiotic resistance genes, as the use of heavy metals may inadvertently co-select for AMR genes (Souza et al., 2022), which further contributed to the successful expansion of this clone (Cadel-Six et al., 2021). The detection of the mercury resistance operon encompassing the mercury resistance genes is in line with the foregoing theory that the chromosomal elements that encode resistance towards mercury in *S.* 4,[5],12:i:- were found associated with certain genetic variations (polymorphisms) involving the

fljBA locus that leads to monophasic phenotype (Elnekave et al., 2018; García et al., 2016; Petrovska et al., 2016). The co-presence of *arsR* and tetracycline resistance genes observed in this study was already described as a result of a co-selection process (Mazhar et al., 2021).

A conserved virulence potential was observed in the isolates under study. The high prevalence of genes associated with *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 shows their ability to successfully invade the host epithelium for infection and their ability to survive and replicate within the host (Jennings et al., 2017; Lou et al., 2019). A prophage-related *sspH2* gene (E3 ubiquitin ligase (NEL) effector) of SPI-2 T3SS that plays a part in E3 ligase activity and causes evasion of the immune response (Jennings et al., 2017; Long et al., 2022) was absent in seven MVST isolates. It seemed predominantly to be a cluster-specific feature, as six isolates that do not carry the *sspH2* gene belonged to cluster II, and only one food strain belonged to cluster I. The detected genomic variations between the two representative strains (*sspH2*-positive and *sspH2*-negative) for the prophage-related *sspH2* gene region suggested that the deleted region of prophage, along with loss of the *sspH2* gene, could have occurred through a process known as prophage decay (in which gradual gene loss occurs over time) (Brüssow et al., 2004). Also, the *sspH2* gene locus can act as a genomic hotspot for prophage-mediated gene deletion and can act as a driver of evolution **(Wahl et al., 2019).** The presence of a genetically stable virulome in all tested MVST samples might contribute to the persistence and spread of this clone, potentially increasing its pathogenicity and posing a significant risk to public health.

Comparative hierarchical cluster analysis of ST34 isolates from our study, alongside the dataset curated by Inge et al. (2021), revealed a population structure with partially very close genetic distances to isolates from our study (7 CE to cluster I isolates and 4 CE to cluster II isolates). Thereby, isolates from our study clustered differently within the ST34 isolates belonging to multiple countries and confirmed the circulation of at least two separate genetic MVST ST34 lineages in Italy (Fig. 3). Analysis hints towards a genetic sub-lineage, predominantly circulating in Italy, including cluster II isolates of our study. Here, we observed a cluster comprising 26 isolates, 22 from Italy (of which 13 Italian isolates belonged to the current study cluster II isolates) and four from the UK. In contrast, ten isolates from this study, belonging to cluster I, clustered closer to ST34 isolates from multiple diverse countries, highlighting the global spread of MVST ST34 isolates through food and travel (Ferrari et al., 2019) and revealing that Italy is also affected by the spread of this widely spread clonal lineage.

This successful dissemination of ST34 isolates in multiple countries signifies its status as a pandemic clone posing severe public health threats (Luo et al., 2023). Hence, it seems, an endemic MVST ST34 sub-lineage (cluster II isolates) with a higher genetic plasticity but partial absence of the *mer* operon exists or might have emerged in the past in Italy, which genetically differs from other successful pandemic MVST ST34 lineages (cluster I isolates).

This study has some limitations. First, the dataset comprised 28 MDR MVST isolates collected through routine surveillance and selected to capture heterogeneity across years and matrices, rather than through a formal random or population-based sampling design. Consequently, the dataset is suitable for exploratory genomic characterization and for identifying circulating lineages, resistance determinants, and possible transmission links, but it does not allow robust inference on the true population structure or prevalence of MVST in the Marche Region. Second, because of the relatively small sample size, rare lineages or less frequent resistance patterns may have remained undetected. Therefore, our findings should be interpreted as a snapshot of the diversity captured within the regional surveillance framework, and future studies based on larger and systematically sampled collections will be necessary to define the regional distribution of MVST with greater precision.

5. Conclusions

The current study provided a detailed characterization of 28 MVST ST34 isolates collected between 2015 and 2021 from multiple sources along the food chain in Central Italy. Phenotypic and genotypic AMR analysis showed a high prevalence of multidrug resistance mainly through chromosomally encoded ASSuT-associated resistance genes, but overall expanded resistance profiles (ASSuT-plus) were dominating the classical ASSuT profile. The cgMLST-based hierarchical clustering analysis grouped isolates mainly into two main clusters, irrespective of strain origin, resistance type, and year of isolation, and suggested a potential transmission of MVST ST34 isolates in the food chain of Central Italy. Distribution of plasmids and virulence genes across clusters also highlights their genetic diversity. A high degree of genetic plasticity was observed in the genetic region responsible for the monophasic phenotype, which was characterized by a high density of IS elements. The combination of a stable set of virulence genes, a diverse array of plasmids, and several AMR and HMRG profiles likely contributes to the transmission and persistence of this clone, highlighting its significant public health implications. Global clustering analysis revealed that there might exist an endemic Italian ST34 subclone showing regional-specific evolution in Italy, being genetically different from other pandemic MVST ST34 lineages, which showed a successful spread among multiple countries. Although these findings should be interpreted in light of the limited and non-randomly selected dataset, they further highlight the importance of continued WGS-based surveillance for monitoring and controlling this MDR MVST ST34 clone, improving our understanding of its emergence and dissemination, and supporting the early detection of additional emerging sub-lineages. Overall, these findings highlight the importance of the One Health approach, which integrates human, animal, and environmental health to understand and mitigate the regional or global spread of this emerging pathogen.

CRediT authorship contribution statement

Jaweria Riaz: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Maria Borowiak:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Maira Napoleoni:** Writing – review & editing, Resources, Methodology. **Giorgio Brandi:** Validation, Supervision, Resources. **Jennie Fischer:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Giulia Amagliani:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.119445.

Data availability

Data will be made available on request.

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