International Scholars Journals

International Journal of Microbiology Research and Reviews ISSN 2329-9800, Vol. 12 (1), pp. 001-010, December, 2024. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Finding Mobile Genetic Components in Salmonella, and Salmonella Typhimurium Serovars that Give Resistance to Heavy Metals and Linking them to Antibiotic Resistance

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Accepted 23 December, 2024

S. 4,[5],12:i:-, also known as Salmonella enterica subsp. enterica serovar Typhimurium variation 4,[5],12:i:-, has quickly become the most common Salmonella serovar in pigs and is frequently linked to the development of heavy metal resistance (HMR) and antibiotic resistance (ABR) genes. Our work examined the evolution of mobile genetic elements (MGEs) comprising HMR and ABR genes in 78 strains of S. 4,[5],12:i:- (n = 57), and S. Typhimurium (n = 21), which were gathered between 1999 and 2021. The following five MGEs were found to harbor HMR genes: pUO-STVR2, pSTM45, pUO-STmRV1, SGI-4, and MREL. 91.23 percent (52/57) of the strains of S. 4,[5],12:i:- carried at least one of these components, while only 14.29 percent (3/21) of the strains of S. Typhimurium did the same. Due to the European Union's ban on the use of antibiotics as feed additives, S. 4,[5],12: i:- have changed from mostly carrying pUO-STmRV1 to the emergence of SGI-4 and MREL, lowering ABR genes. SGI-4 and MREL imparted increased resistance to copper and silver in S. 4,[5],12:i:-, indicating that their acquisition was associated with the continued use of heavy metals in food-animal production. The necessity for focused interventions to reduce the spread of multidrug-resistant Salmonella in veterinary and public health settings is highlighted by the fact that strains bearing SGI-4 and MREL still show multidrug resistance.

Key words: S. 4,[5],12:i:-, S. Typhimurium, Antibiotic resistance (ABR), Heavy metal resistance (HMR), Plasmid Genomic island.

INTRODUCTION

One of the most common serovars in humans in the European Union (EU) and the first most frequently reported serovar in pigs is the monophasic strain of Salmonella Typhimurium (S. 4,[5],12:i:-) (EFSA and ECDC, 2023). The establishment of this variant has been linked to the acquisition of heavy metal resistance (HMR) and antibiotic resistance (ABR) genes since the first strains were reported (Echeita et al., 2001; Laorden et al., 2010; Cadel-Six et al., 2021; Petrovska et al., 2016).

Antibiotics have been used for decades to enhance animal growth in animal production, as is well known (Chattopadhyay, 2014). However, the EU outlawed the use of antibiotics as growth promoters in feed in 2006 due to the increase in ABR (European Parliament and the Council of the European Union, 2003). As an alternative, a number of heavy metals with antibacterial properties, like copper and silver, are currently frequently utilized in animal production in the EU as disinfectants or as feed additives, respectively (Argudín et al., 2019; European Commission, 2022; SCENIHR, 2014). Depending on the particular animal group, the maximum limitations for copper as a feed addition are

either 150 mg/kg or 100 mg/kg (European Commission, 2018). Furthermore, quantities of ionized silver ranging from 0.1 to 20 ppm and concentrations of silver nanoparticles ranging from 1 to 50 ppm are permitted for use in disinfection products (European Parliament and the Council of the European Union, 2020). However, from the middle of the 1940s, various heavy metals and their derivatives—like organic arsenic compounds—have been used as feed additives. In 1999, the EU outlawed them due to the hazards they posed to public health (EFSA, 2005; Council of the European Union, 1999). Along with pollutants like mercury, arsenic has been recognized as one of the undesirable compounds in animal feed since 2002 (Dorne et al., 2013).

Crucially, metals can accumulate in agricultural and animal production settings and are not degraded (Yu et al., 2017). When the respective resistance genes are located in the same mobile genetic element (MGE), metals can play a significant role in the co-selection of bacteria that are resistant to both metals and antibiotics (Baker-Austin et al., 2006; Seiler and Berendonk, 2012). To date, a number of MGEs that contain both ABR and HMR genes have been identified in S. 4,[5],12:i:-, and in its closest evolutionary predecessor, S. Typhimurium. For example, in 2002, it was discovered that S. Typhimurium harbored the plasmid pUO-STVR2, which contained multiple ABR genes (aadA1, blaOXA-1, catA1, sul1, and tet(B)) and mercury resistance genes (Antunes et al., 2004; Guerra et al., 2002; Herrero et al., 2008a; Herrero et al., 2008b). Since then, strains from human and swine infection cases from several European nations have been found to carry the plasmid (Bances et al., 2007; Beutlich et al., 2013; Herrero et al., 2009).

The plasmid pUO-STmRV1 (formerly pUO-SVR3), on the other hand, was described in S. 4,[5],12:i:- in 2001 and had a higher degree of diversity in HMR genes (Guerra et al., 2001). This plasmid had genes that conferred resistance to four metals, namely arsenic (arsHR2), mercury (merRTPCADE), and silver and copper (silESRCBAP), as well as commonly used antibiotics (Va´zquez et al., 2021). Additionally, the second phase flagellar region and other adjacent loci of S. 4,[5],12:i:- ST34 strains that were isolated from humans, livestock, and contaminated food were found to contain a composite transposon called the Mercury Resistance Element (MREL) (Clark et al., 2020; Lucarelli et al., 2012; Petrovska et al., 2016). Together with ABR genes including blaTEM-1, strB, strA, sul2, and tet(A), this element encodes mercury resistance (Lucarelli et al., 2012). In S. 4,[5],12:i:- strains, the Salmonella genomic island 4 (SGI-4), which codes for HMR genes against copper, silver, and arsenic, was often found concurrently with MREL (Branchu et al., 2019). Porcine and clinical samples were the primary sources of SGI-4 detection (Clark et al., 2020; Petrovska et al., 2016; Va´zquez et al., 2021, 2023).

Significant factors affecting bacterial evolution in pig production systems include the buildup of heavy metals in the environment and the co-selection of Salmonella that carry both the HMR and ABR genes. Spain, a significant pork producer in the EU and the third-largest producer in the world after China and the USA, makes these elements very intriguing to look at (MAPA, 2021). The lack of an official Salmonella control program in pig production (Martínez-Avil´es et al., 2019; Teng et al., 2020) has resulted in a lack of knowledge regarding the evolution of Salmonella strains in pigs and their subsequent impact on human clinical cases in Spain, despite the country's notable prevalence of breeding pig farms that are positive for the pathogen. In order to comprehend the potential selective advantage that these elements may offer to S. 4,[5],12:i:- emerging clones circulating in Spain in contrast to S. Typhimurium biphasic clones, this study sought to analyze the temporal distribution and evolution of MGEs harboring ABR and HMR genes.

2. Material and methods

2.1. Strain collection and whole-genome sequencing (WGS)

id, Spain), the Institute of Agrobiotechnology-CSIC (Mutilva, Navarra, Spain), the National Centre for Microbiology (Majadahonda, Madrid, Spain), or the Public Health Laboratory of the Basque Government (Derio, Vizcaya, Spain) after being serotyped using the Kauffmann–White scheme (Grimont and Weill, 2007). Following the manufacturer's instructions, the NucleoSpin Tissue DNA purification kit (Macherey-Nagel, Duren, Germany) was used to extract and purify the genomic DNA from the 78 strains. The Illumina MiSeq and Illumina NovaSeq platforms (SGIker, Spain and Eurofins Genomics, Germany) were used to sequence the genomes. Fastp was used to analyze the quality of the raw reads (Chen et al., 2018); Trimmomatic v0.39 was used to trim the reads (Bolger et al., 2014); and SPAdes v3.15.2 was used to assemble the trimmed reads into contigs (Prjibelski et al., 2020). The NCBI received assembled genomes as part of BioProject PRJNA1066061. The pubMLST database (https://github.com/tseemann/mlst) was used to determine the multilocus sequence typing (MLST) profiles (Jolley et al., 2018).

2.2. Detection and localisation of HMR and ABR genes

AMRFinderPlus v3.11 was used to screen the entire genomes for the presence or lack of ABR and HMR genes against arsenic, copper, mercury, and silver (Feldgarden et al., 2021). ArsH (accession CP018220, region 68718-69428) and arsR2 (accession CP018220, region 68302-68625) sequences were searched using ABRICATE (https://github.com/tseemann/abricate) in order to finish the detection of the HMR genes.

To confirm their relationship with chromosomes or plasmids, contigs containing HMR and ABR genes were searched using MOB- suite v3.1.7 (Robertson and Nash, 2018) and Mash v2.3 (Ondov et al., 2016). Lastly, the entire genome of each strain was compared with the complete sequence of

the nearest neighbor element using BLASTN v2.14.0 (J. Chen et al., 2015). If the coverage and identity with reference sequences were greater than 80% and 90%, respectively, it was considered that MGEs were present.

2.3. Reconstructing ancestral states and time-scaled phylogeny

Snippy v4.6.0 (Seemann, 2015) was used to identify Single Nucleotide Polymorphisms (SNPs) using the trimmed reads as input files. The reference genome was that of the S. Typhimurium LT2 strain (GeneBank accession number AE006468.2). A time-scaled phylogenetic tree and ancestral state reconstructions based on the Bayesian technique using BEAST v1.10.4 were constructed using the core SNP alignment and the isolation date of each strain (Drummond and Rambaut, 2007). The Coalescent Exponential Population model (Kingman, 1982) and the uncorrelated relaxed clock (Drummond et al., 2006) were employed for this purpose.

2.4. Heavy metals susceptibility test

Using Mueller-Hinton agar plates (Scharlau) and the following compounds and concentrations, the agar dilution method was used to determine the minimum inhibitory concentrations (MICs) of copper, silver, mercury, and arsenic: CuSO4 2–16 mM (Panreac), AgNO3 0.06–1.00 mM (Probus), HgCl2 4–64 µg/mL (Panreac), and C6AsNH6O6 1024–8192 µg/mL (roxarsone, ABCR GmbH). The supplemented plates were incubated at 37 ◦C for 24 hours under aerobic circumstances for AgNO3, HgCl2, and roxarsone, and for 48 hours under anaerobic conditions for CuSO4 (Arai et al., 2019). In short, 10 µL of a 1.5 × 108 CFU/mL suspension of each strain was injected onto the plates as dots. In every assay, S. Typhimurium ATCC 14028 served as the control strain. Three biological replicates and triplicate runs of the experiments were conducted.

2.5. Statistical analyses

The Shapiro-Wilk test was used to evaluate the variables' normal distribution. Non-parametric variables were statistically compared using the Mann-Whitney pairwise U test. The Pearson correlation coefficient was used to evaluate the relationship between the ABR and HMR genes. Linear regression was used to evaluate the relationship between the number of ABR genes and the strains' isolation date. R v4.3.1 was used for all statistical analyses and visualizations (R Core Team, 2021).

3. Results

3.1. Prevalence and localization of HMR genes

HMR genes against at least one heavy metal were found in 91.23% (52/57) of the 78 strains that were examined, including S. 4,[5],12:i:-, and 14.29% (3/21) of S. Typhimurium strains (Table A2). No HMR genes against copper, arsenic, silver, or mercury were found in the remaining strains. Nine strains out of the entire collection had two MGEs at the same time (Table 1). HMR genes were discovered in MGEs that have previously been reported in the literature (>80% coverage): pUO-STmRV1 plasmid (n = 15), MREL transposon (n = 8), pUO-STVR2 plasmid (n = 2), pSTM45 plasmid (n = 1), and SGI-4 genomic island (n = 28 strains). Ten additional S. 4,[5],12:i:-strains, however, showed 66–74% coverage of the pUO-STmRV1 plasmid sequence, with the exception of a 30–35 kb segment. These strains were therefore categorized as having a partial pUO-STmRV1 plasmid.

SilABCEFPRS, which confers resistance against copper and silver, was the most often found resistance operon. In 15 strains, this operon was found inside the entire pUO-STmRV1 plasmid sequence; in 28 strains, it was found within the SGI-4; and in one strain, it was found within the pSTM45. Resistance to mercury was conferred by the second most prevalent operon found. ABDGPRT was found in pSTM45 (n = 1), while merCPRT was found in the full sequence of pUO-STmRV1 (n = 15), in the partial sequence of pUO-STmRV1 (n = 10), in the pUO-STVR2 (n = 2), and in MREL (n = 8). Additionally, several arsenic resistance genes were found, including arsABCDR in the SGI-4 (n = 28), arsHR2 in the full and partial sequences of pUO-STmRV1 (n = 15 and 10), and arsC in pSTM45 (n = 1). Lastly, the SGI-4 (n = 28) and the pSTM45 (n = 1) were found to have the pcoACDRS operon and the pcoS gene, which give resistance to cop-per, respectively.

3.2. Prevalence of ABR genes and association with elements harbouring HMR genes

The strains that were examined showed a considerable degree of variation in their ABR genes. Thirty-one different combinations of ABR genes were found out of the 34 different ABR genes that were identified (Table A2). Each strain had at least two ABR genes, but the most common ones were blaTEM-1 (n = 33), which encodes beta-lactam resistance; sul2 (n = 46), which encodes sulphonamide resistance; aadA2 (n = 39), which encodes aminoglycoside resistance; and mdsA and mdsB (n = 76), which encode a multidrug efflux pump that confers resistance to novo-biocin and other toxic compounds. Crucially, three strains were found to have clinically significant ABR genes, including mcr-1.32, mcr-5.1, and mcr-9.2, which encode colistin resistance, and blaCTX-M-9, a broad-spectrum beta-lactamase.

The MGEs harboring the HMR genes identified in this investigation contained the majority of the observed ABR genes. As previously reported in S. Agona and S. 4,[5],12:i:-, respectively, a subset of the ABR genes were found in

different chromosome regions (mdsA and mdsB) and in plasmids that did not contain HMR genes, such as pSAG-76,334 (GenBank: CP025455.1) and pST34VN3 (GenBank: OQ658821.1). Interestingly, 20 of the 34 ABR genes found in this study exhibited a substantial positive association (p value < 0.05 and Pearson correlation coefficient > 0.22) with the MGEs that contained HMR genes (Fig. 1). 11 ABR genes, including aac(3)-IVa, aadA1, aadA2; blaTEM-1, bleO, cmlA1, dfrA12, sul1, sul2, sul3, and tet(A), were linked to the full pUO-STmRV1 plasmid sequence. Together with HMR genes that protect against silver, copper, mercury, and arsenic, all of these genes were found on the pUO-STmRV1 plasmid. The relationship with the aac(3)-IVa, bla-TEM-1, bleO, and dfrA12 genes was eliminated upon detection of the incomplete pUO-STmRV1 plasmid. pSTM45 and pUO-STVR2 sequences were linked to distinct ABR genes, respectively. The latter has aadA1, blaOXA-1, catA1, and tet(B), while the former has blaCTX-M-9, drfA16, and mcr-9.2. Together with HMR genes, these genes were precisely found on the aforementioned plasmids. Finally, the presence of SGI-4 in the strains under study was linked to the presence of three ABR genes against aminoglycosides: strA, strB, and aph(4)-Ia. This is despite the fact that the genomic island SGI-4 itself does not include ABR genes. The three resistance genes in this instance were discovered to be located on different plasmids, including pSAG-76334 and pST34VN3. An relationship with two other ABR genes found in the MREL (blaTEM-1 and tet(B)) was noted when the MREL was also found in strains with SGI-4.

3.3. Temporal distribution and evolution of the elements harbouring HMR genes and ABR genes in S. 4,[5],12:i:- and S. Typhimurium

Based on the dates of isolation of the S. 4,[5],12:i:- strains, two separate clusters were found, each of which contained unique components harboring HMR and ABR genes, as illustrated in Fig. 2. On the one hand, pUO-STmRV1 was only found in S. 4,[5],12:i:-ST19 strains, and it was primarily isolated between 1999 and 2006. It was found that 15 S. 4,[5],12:i:- strains carried the entire pUO-STmRV1 plasmid sequence. However, the partial sequence of the pUO-STmRV1 plasmid was found in 10 more S. 4,[5],12:i:-strains. The 30–35 kb segment that contained the resistance operon silABCEFPRS and four ABR genes (aac(3)-IVa, blaTEM-1, bleO, and dfrA12) was absent from this incomplete plasmid. The ancestral state reconstruction indicated that there was a 77.34% chance that the strains' common ancestor carried the entire pUO-STmRV1 plasmid before the loss of the four ABR genes and the silver/copper resistance operon in an individual event or events.

However, the SGI-4 island acquisition was noted starting in 2008 and continued in 26/27 S. 4,[5],12:i:-ST34 strains as well as in the single S. 4,[5],12:i:-ST8802 strain that was isolated until 2021. Additionally, a phylo-temporal link between the S. Typhimurium ST34 strain, which carries both plasmid pSTM45 and SGI-4, and the S. 4, [5],12:i:-ST34 strains was noted. As previously stated, it was discovered that eight S. 4,[5],12: i:-strains simultaneously had the MREL and the genomic island SGI-4. The ancestral state reconstruction analysis also revealed that the acquisition of MREL was a later genetic event, with a 63.15 percent chance that the common ancestor of these S. 4,[5],12:i:-ST34 strains initially gained SGI-4. Lastly, a close phylogenetic link between the two ST19 S. Typhimurium strains carrying the plasmid pUO-STVR2 and the S. Typhimurium ST19 strains devoid of metal resistance genes was observed.

Number of strains

Mobile genetic elements (HMR genes)

A substantial negative connection (p < 0.001) was found between the two variables during the isolation period of the strains under study. This pattern showed that the more recent the isolation date, the fewer ABR genes were detected (Fig. 3). In particular, the average number of ABR genes was 12.53 ± 0.92 and 9.20 ± 1.93 among the S. 4,[5],12:i:-strains that included the full and partial sequencing of the pUO-STmRV1 plasmid, which constituted the oldest strains in this investigation. The most recent strains of S. 4,[5],12:i:-, and S. Typhimurium, which simply carried the SGI-4, a combination of SGI-4 and MREL, or the pUO-STVR2 plasmid, showed noticeably less ABR genes. The average ABR gene levels for these strains were 6.11 ± 2.92, 7.25 ± 1.67, and 7 ± 0. The most recent S. 4,[5],12:i:-ST34 strain, which was identified in 2021 and displayed the SGI-4 island along with 14 ABR genes, is an exception that should be noted. These genes, however, were linked to the plasmid pST34VN3, which was found exclusively in this strain, rather than any element that contained HMR genes.

3.4. Heavy metal susceptibility

In the heavy metal susceptibility tests for the four chemicals examined, strains lacking HMR genes exhibited the lowest MIC values (Fig. 4).

Higher MIC values against CuSO4 were observed in strains carrying the HMR genes against copper, namely SGI-4, pSTM45, and pUO-STmRV1 (Fig. 4A). In comparison to cultures lacking copper resistance genes (4 mM), the majority of SGI-4 strains (85.71 %, p < 0.001) and 26.66% of strains with the entire pUO-STmRV1 (p = 0.074) had two to three times higher MIC values (8–12 mM).

For AgNO3, a similar pattern was seen. SGI-4, pSTM45, and full pUO-STmRV1 were MGEs that carried HMR genes against silver. The majority of bacteria with pSTM45 and the SGI-4 genomic island (82.14 percent, p < 0.001) had MIC values against silver (0.50 mM) that were four times higher than those of strains without HMR genes (0.06 mM) (Fig. 4B). However, bacteria using the whole pUO-STmRV1 plasmid's silABCEFPRS operon showed less resistance to AgNO3 (0.13 mM).

Resistance to HgCl2 was imparted by MGEs with HMR genes against mercury (pUO-STmRV1, pSTM45, pUO-STVR2, and MREL). The strains with these elements had a four-fold higher MIC value (32 μg/mL) against mercury than strains without HMR genes in the majority of cases (91.67%, $p < 0.001$) (Fig. 4C).

Last but not least, cultures that carried MGEs with HMR genes against arsenic (pUO-STmRV1, pSTM45, and SGI-4) showed MIC values against roxarsone that were two times higher (4096 μg/mL, $p < 0.001$) than strains that did not carry them (2048 μg/mL) (Fig. 4D).

4. Discussion

Multidrug-resistant strains, especially S. 4,[5],12:i:-, have been selected and expanded as a result of the extensive use of heavy metal compounds in food-animal production, which has been suggested as a possible co-selection factor impacting bacterial evolution (Cadel-Six et al., 2021; Mastrorilli et al., 2018; Moura˜o et al., 2015). In order to explore the evolution of MGEs including HMR and ABR genes, we looked at S. 4,[5],12:i:- and its closest evolutionary relative, S. Typhimurium.

Sequences of five distinct MGEs including HMR genes were found in the complete collection under analysis: pUO-STVR2, pSTM45, pUO-STmRV1, SGI-4, and MREL. Out of the 21 strains of S. Typhimurium, only three showed one of these MGEs associated with HMR genes. The plasmid pUO-STVR2 sequence was found in two S. Typhimurium ST19 strains, containing five ABR genes (aadA1, blaOXA-1, catA1, sul1, and tet(B)) as well as genes for mercury resistance. Since it has been found before, the presence of the pUO-STVR2 plasmid in S. Typhimurium is not a unique occurrence in Spain (Herrero et al., 2006; Herrero et al., 2008a; Herrero et al., 2008b). Furthermore, strains of this plasmid have been found in human infection cases in Italy and the UK as well as in pigs (Beutlich et al., 2013; Herrero et al., 2009), suggesting that the plasmid is spreading throughout Europe. The third S. Typhimurium strain that tested positive was of sequence type ST34 and carried the plasmid pSTM45 in addition to SGI-4. Specifically, this strain was reported in one of our earlier investigations, in which we described pSTM45 as a 300 kb plasmid with a novel antimicrobial resistance region that carries HMR genes and clinically relevant ABR genes, including the colistin resistance gene mcr-9 and the extended-spectrum β-lactamase blaCTX-M-9 (Garrido et al., 2024).

As far as we are aware, this plasmid has not yet been discovered in any other strain globally, even though the strain was isolated in 2012.

Unlike S. Typhimurium strains, at least one MGE containing HMR genes was present in 91.23 percent of S. 4,[5],12:i:- strains. In particular, 19 strains carried the SGI-4, eight strains carried SGI-4 in conjunction with the MREL, and 25 S. 4,[5],12:i:- strains carried the full or partial pUO-STmRV1 plasmid sequence. The historical analysis revealed a clear evolution of MGEs with HMR genes in the strain collection under study. The pUO-STmRV1 plasmid sequence was present in the first isolated S. 4,[5],12:i:-ST19 strains in our investigation. The plasmid pUO-STmRV1 evolved through independent genetic events, losing the silABCEFPRS operon and four ABR genes (aac(3)-IVa, blaTEM-1, bleO, and dfrA12), according to ancestral state reconstruction. This led to the partial pUO-STmRV1 plasmids found in 10 S. 4 [5],12:i:- ST19 strains in our investigation. In contrast to cultures with other MGEs, susceptibility experiments revealed that bacteria containing the silABCEFPRS operon in full pUO-STmRV1 demonstrated resistance to extremely low amounts of silver. Therefore, in a variety of S. 4,[5],12:i:- strains, the deletion of the silABCEFPRS operon from the pUO-STmRV1 plasmid may represent a dynamic process influenced by selective pressures, resulting in the loss of genetic material that confers low-level resistance. Whether the pUO-STmRV1 plasmid has been detected in S. 4,[5],12:i:-strains isolated from Spain is still unknown. According to some research, strains of S. 4,[5],12:i:- in Portugal, France, and the UK may have "pUO-STmRV1-like" plasmids harboring pUO-STmRV1-like integrons and transposons (Antunes et al., 2011; Bugarel et al., 2012; García et al., 2014).

Our strain collection's MGEs carrying HMR genes clearly evolved over time, according to the temporal study. The introduction of SGI-4 and MREL in S. 4,[5],12:i:- ST34 may replace the presence of pUO-STmRV1 in S. 4,[5],12:i:-ST19 isolated before to 2008. SGI-4 is a conjugative, self-transmissible element, as previous research has shown (Arai et al., 2019; Branchu et al., 2019). According to Petrovska et al. (2016), it was suggested that SGI-4 might have started by incorporating a plasmid sequence into the chromosome. To yet, however, no characterized plasmid has been connected to the precise origin of SGI-4 (Clark et al., 2020; Petrovska et al., 2016). However, despite the presence of plasmid-derived genes in MREL (García et al., 2016; Lucarelli et al., 2012), conjugation tests failed to transfer MREL from the donor strain to the recipient strain, suggesting that horizontal transfer is not possible (Lucarelli et al., 2010). The genomic organization of SGI-4 and MREL is different from the plasmid sequences found in the Salmonella strains used in this investigation, namely pUO-STmRV1, pSTM45, and pUO-STVR2, even though they share the majority of HMR genes. This implies that SGI-4 and MREL most likely evolved from other sources rather than from these particular plasmids.

SGI-4 and MREL were acquired in the late 1980s and 1982, respectively, according to a genomic research on the epidemic clone ST34 conducted by Denmark, France, Germany, Italy, and the UK (Cadel-Six et al., 2021). However, prior to 2008, our investigation could not find any strains of S. 4,[5],12:i:-ST34 with SGI-4 and MREL. This may be because S. 4,[5],12:i:-ST19 with pUO-STmRV1 was quite common in Spain at the time antibiotics were utilized as feed additives. It may have occupied the ecological niche because of ABR provided by the pUO-STmRV1 plasmid. Our study's sampling of S. 4,[5],12:i:- coincided with the EU's gradual implementation of the ban on antibiotics as feed additives from 2003 to the start of 2006 (European Parliament and the Council of the European Union, 2003).

The rise of S. 4,[5],12:i:-ST34, which harbored the SGI-4 and MREL islands linked to fewer ABR genes, replaced the high predominance of S. 4,[5],12:i:-ST19, which harbored pUO-STmRV1. This implies that the prohibition on the use of antibiotics as feed additives was successful in lowering ABR, which helped S. 4,[5],12:i:-evolve, with MGEs having less ABR genes. But as this study points out, the strains of S. 4,[5],12:i:-ST34 that were currently identified and harbored SGI-4 and MREL were still linked to a number of ABR genes, including aph(4)-Ia, bla-TEM-1, strA, strB, and tet(B). Furthermore, although there are outliers, it should not be believed that the decline in ABR genes is widespread. For example, the strain of S. 4,[5],12:i:-ST34 that was isolated in 2021 had the ABR profile with the greatest number of resistance genes (n = 14) found in this investigation, as well as the SGI-4 island. Most of these ABR genes belonged to the incHI2 incompatibility group and were found in the plasmid pST34VN3 (Chung The et al., 2023).

Trends in heavy metal susceptibility and substitution on MGEs with HMR genes are consistent with real-world uses in livestock environments. In contrast to the older S. 4,[5],12:i:-ST19 strains with either complete or partial pUO-STmRV1, the most recent strains of S. 4,[5],12:i:-ST34, which carry the islands SGI-4 and MREL, maintained a similar MIC against mercury and arsenic but showed a higher MIC against silver and copper. The resistance capacity did not significantly evolve despite changes in the location of genes in different MGEs carrying HMR genes, which makes sense given that mercury and arsenic were recognized as undesirable contaminants in animal feed (Council of the European Union, 1999) since the initial isolation of Salmonella strains in this study in 1999. However, the acquisition of the SGI-4 island by recent isolated S. 4 [5],12:i:-ST34 strains showed greater resistance to copper and silver, which was consistent with the continued use of these metals in food-animal production (Bearson et al., 2020; Branchu et al., 2019).

Notably, the type of arsenic examined in this study was roxarsone, a substance that has long been added to animal feed to prevent coccidiosis, increase feed efficiency, and encourage weight gain (Liu et al., 2022). Resistance to the organic As(V) compound roxarsone is not directly conferred by the genes arsABCDR and arsHR2, which are found on SGI-4 and pUO-STmRV1, respectively. The MIC value of the bacteria in our investigation that had these HMR genes, however, was double that of the ones that did not. This might occur as a result of the formation of inorganic arsenic due to roxarsone's susceptibility to abiotic breakdown via photolysis (Meng et al., 2022). Thus, in light-exposed experiments, roxarsone may break down into inorganic forms that the arsABCDR system (encoded by SGI-4) can manage (Ben Fekih et al., 2018). Furthermore, organisms bearing the pUO-STmRV1 plasmid showed a fourfold increase in resistance to roxarsone in comparison to control strains in the study by Va´zquez et al. (2021) S. 4,[5],12:i:-. The arsHR2 genes may possibly be responsible for this increased resistance. Indeed, decreased roxarsone was shown to be detoxified by the enzyme that arsH encodes (Y. Chen et al., 2015).

5. Conclusions

All things considered, our research offers a thorough examination of the dynamics between MGEs that carry ABR and HMR genes as well as the development of the multidrug-resistant S. 4,[5],12:i:-. The cause of its origin is supported by the remarkable diversity of MGEs found in S. 4,[5],12:i:-. With the development of SGI-4 and MREL in S. 4,[5],12:i:- ST34 strains displacing the formerly dominating pUO-STmRV1 in ST19 strains, time-scaled phylogeny shows a significant evolution on MGEs containing HMR genes, indicating a reaction to changes in antibiotic usage policies. This shift is accompanied by the EU's ban on antibiotics as feed additives, which causes strains with fewer ABR genes overall to arise. Notably, the acquisition of the SGI-4 island is responsible for the improved resistance to copper and silver in S. 4,[5],12:i:-ST34 strains, which is consistent with real-world methods in food-animal production. This study highlights the connections between metal susceptibility patterns and the evolution of S. 4, [5],12:i:-, and demonstrates a strong association between the existence of HMR and ABR genes. In both veterinary and public health contexts, the results emphasize the significance of focused interventions to reduce the emergence and spread of Salmonella strains that are resistant to several drugs.

Funding

This work was financially supported by the Basque Government (grant number PA20/03) and the University of the Basque Country (UPV/EHU) (grant number GIU21/021). A. Arrieta-Gisasola is recipient of a Ph.D. fellowship from the University of the Basque Country (UPV/ EHU) (PIF 19/290).

Ethical approval

Not required.

Declaration of competing interest

The authors state that none of the work described in this study could have been influenced by any known competing financial interests or personal relationships.

Data availability

Data will be made available on request.

Acknowledgments

We appreciate the helpful cooperation on Salmonella serotyping from the Public Health Laboratory of the Basque Government (Derio, Vizcaya, Spain), the Central Veterinary Laboratory (Algete, Madrid, Spain), and the National Centre for Microbiology (Majadahonda, Madrid, Spain).

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