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# Genome analysis of antimicrobial resistance, virulence, and plasmid presence in Turkish *Salmonella* serovar Infantis isolates

Sinem Acar[a], Ece Bulut[b], Matthew J. Stasiewicz[c], Yeşim Soyer[a,*]

[a] Department of Food Engineering, Middle East Technical University, Ankara 06810, Turkey
[b] Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68588, USA
[c] Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

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ABSTRACT

*Salmonella enterica* subsp. *enterica* serovar Infantis (*S*. Infantis) isolates were found to have a multi-drug resistance profile (kanamycin, streptomycin, nalidixic acid, tetracycline, sulfonamide, and sometimes to ampicillin) and high prevalence (91%) in Turkish poultry in our previous studies. To investigate the mechanism behind multi-drug antimicrobial resistance (AMR) and high prevalence in Turkish poultry, 23 of the isolates were sequenced for comparative genomic analyses including: SNP-based comparison to *S*. Infantis from other countries, comparison of antimicrobial resistance genes (AMGs) with AMR phenotypes, and plasmid identification and annotation. Whole-genome SNP-based phylogenetic analysis found that all 23 Turkish *S*. Infantis isolates formed a distinct, well-supported clade, separate from 243 comparison *S*. Infantis genomes in GenomeTrakr identified as from the US and EU; the isolates most closely related to the cluster of these Turkish isolates were from Israel and Egypt. AMGs identified by bioinformatic analysis, without differentiating chromosomal or plasmid located genes, implied AMR phenotypes with 94% similarity overall to wet lab data, which was performed by phenotypic and conventional PCR methods. Most of the *S*. Infantis (21/23) isolates had identifiable plasmids, with 76% (16/21) larger than 100 kb and 48% (10/21) larger than 200 kb. A plasmid larger than 200 kb, with the incompatibility type of IncX1, similar to United States *S*. Infantis plasmid N55391 (99% query coverage and 99% identity overall), which itself is similar to Italian and Hungarian *S*. Infantis plasmids. Turkish *S*. Infantis plasmids had different beta-lactam resistance genes ($bla_{\text{TEM-70}}$, $bla_{\text{TEM-148}}$ and $bla_{\text{TEM-198}}$) than the gene $bla_{\text{CTX-M-65}}$ found in *S*. Infantis plasmids from other countries. This is the first observation of these three genes in *S*. Infantis isolates. The plasmids larger than 200 kb had two distinct regions of interest: Site 1 and Site 2. Site 1 (around 130 kb) had virulence- and bacteriocin- associated genes such as bacteriocin secretion system and type II toxin-antitoxin system genes (*vagC*, *ccdA*, *ccdB*, *mchE*, *cvaB*) and an aminoglycoside resistance gene (*str*). Site 2 (around 75–110 kb) had the antimicrobial resistance genes (*aadA*, *sulI*, *tetA*, *tetR*) and mercury (*mer*) resistance gene on tranposons Tn552 and Tn501. Presence of these AMR and virulence genes suggests they may have a role in the emergence of *S*. Infantis in poultry and support treating this serotype as a an important human health hazard.

## 1. Introduction

Non-typhoidal *Salmonella enterica* subsp. *enterica* (*Salmonella*) is one of the most important causes of human foodborne illness worldwide (EFSA&ECDC, 2015; Hendriksen et al., 2011; Scallan et al., 2015). Globally, *Salmonella* is estimated to cause 80.3 million food-borne gastroenteritis cases in both developing and developed countries (Majowicz et al., 2010). Although there are over 2600 serovars of *Salmonella*, a few of them are the most common in humans, including *Salmonella* serovar Infantis (*S*. Infantis) (Hauser et al., 2012). From 2003 to 2013 in the US, the laboratory-confirmed incidence of *S*. Infantis infections in humans reported to CDC has increased from 570 to 1342 annually, with an average of 816 cases (CDC, 2016). With the recent increase in the incidence of *S*. Infantis cases, the serovar was ranked as the sixth most frequent serovar causing the among 7452 confirmed human cases of salmonellosis in the US in 2014 (Crim et al., 2015). Although US *S*. Infantis outbreaks are associated mostly with poultry and pork (CDC, 2011), some of the outbreaks were related to other sources such as grains, beans, beef and dry dog food, indicating diversity of its presence in foods (Shah et al., 2017). Similarly, in the

* Corresponding author.
*E-mail address:* ysoyer@metu.edu.tr (Y. Soyer).

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European Union (EU), the number of *S*. Infantis isolates reported in 2014 was more than twice as many reported in 2010. The EU ranked Infantis as the fourth most common *Salmonella* serovar found in human cases, with 1846 confirmed human cases in 2014 (EFSA&ECDC, 2015). Moreover, *S*. Infantis is reported as the most commonly found serovar in chicken and turkey, as well as chicken meat, often with a multidrug resistance (MDR) profile (Nógrády et al., 2012). Many parts of the world reported MDR *S*. Infantis strains from poultry and human sources, indicating that *S*. Infantis may be an emerging public health problem internationally (Asgharpour et al., 2014; Gal-Mor et al., 2010; Pessoa-Silva et al., 2002; Shahada et al., 2010a, 2010b; Velhner et al., 2014). In Turkey where *S*. Infantis is the forth common serovar among human cases, > 90% of the *Salmonella* isolates obtained from human cases had multi-drug resistance profiles attributed to plasmids > 150 kb, showing the significance of MDR Infantis in Tukey (Ozdemir and Acar, 2014).

*S*. Infantis isolates, especially from meat sources, generally have concerning antimicrobial resistance profiles. An increase of multi-drug resistance (MDR) in *S*. Infantis in broiler chicken and meat had been reported in Italy, Hungary and Israel (Aviv et al., 2016; G. Aviv et al., 2014; A. Franco et al., 2015; Nógrády et al., 2012; Nógrády et al., 2007). Resistance to nalidixic acids, streptomycin, sulfonamides, tetracyclines, extended-spectrum beta lactamases were detected in *S*. Infantis isolates and MDR was frequently found to be associated with conjugative mega-plasmids (approximately 280 kb). Since *Salmonella* Infantis is one of the leading serovars observed in humans, foods and animals in Europe (EFSA, 2015), the mechanisms behind these prevalent and widely-spread antimicrobial resistance profiles should be investigated and their spread should be monitored.

Novel genetic traits are acquired in bacteria by either mutations or horizontal gene transfer. Horizontal gene transfer enables bacteria to utilize mobile genetic elements from outside, like plasmids, to express various phenotypes, including antimicrobial resistance (AMR) (Wozniak and Waldor, 2010). Horizontal transfer of AMR carrying plasmids is one of the predominant ways for pathogens to transfer and acquire AMR genes, due to plasmids' potentially broad host range and conjugative ability to facilitate transfer between organisms (Carattoli, 2013; Thomas and Nielsen, 2005). Studies around the world revealed that the plasmids of *S*. Infantis strains confer resistance to major classes of antibiotics like β-lactams, cefotaximes, ciprofloxacins, cephalosporins, tetracyclines, sulfonamides, fluoroquinolone, and trimethoprims, often simultaneously (Gili Aviv et al., 2014; Cloeckaert et al., 2007; Colobatiu et al., 2015; Franco et al., 2015; Kehrenberg et al., 2006; Shahada et al., 2010).Therefore, understanding properties of AMR gene conferring plasmids are important to developing appropriate surveillance for AMR spread.

With the development of reasonably priced whole genome sequencing (WGS) technology, it is possible to detect and analyze the nearly complete DNA sequence in few days, which makes WGS a powerful surveillance tool (Leekitcharoenphon et al., 2014), particularly in epidemiological studies when used for bacterial typing. Differentiation of very closely related isolates becomes more powerful with WGS compared to pulsed field gel electrophoresis (PFGE), limited-loci multilocus sequence typing (MLST) or multiple-locus variable number tandem repeat analysis (MLVA) and other molecular subtyping methods because the core genome can be assessed through SNP-based or MLST methods, which determine evolutionary relationships. Also, WGS analysis enables the identification of characteristics which cannot be obtained with other typing methods (Allard et al., 2012). With sufficient genomic data, it is possible to discover the critical genes that play role in pathogenesis, antimicrobial resistance, carbon source usage, and plasmid content (Gardy et al., 2011; Holt et al., 2008). These analyses allow a more rigorous analysis of diversity and relatedness (Wilson et al., 2016). These analyses of WGS data have been applied to study the molecular mechanism of *Salmonella* virulence and abundance (Jagadeesan et al., 2018; Hindermann et al., 2017).

Our earlier study (Acar et al., 2017) presented antimicrobial resistance profiles of *S*. Infantis isolates obtained from chicken meat in Turkey and found MDR profiles were similar to previous European studies. In addition, every *S*. Infantis isolate ($n = 50$) which was isolated from chicken had phenotypic MDR profiles. This result led to the question of the mechanism of antimicrobial resistance in these S. Infantis isolates. This current study uses WGS for comparative analysis of the core genome phylogenetic structure and presence and organization of antimicrobial resistance genes and plasmids to understand the mechanism of MDR profiles of these Turkish *S*. Infantis isolates. We also identified virulence factors.

## 2. Materials and methods

### 2.1. Bacterial isolates

All of the 23 *Salmonella* Infantis isolates used in this study were collected from the same region, Şanlıurfa, Turkey from April 2012 to January 2013 in a previous study (Durul et al., 2015). They were obtained from chicken meat sources according to ISO 6579 procedures performed at METU in Ankara and serotyped by White- Kauffmann-LeMinor Scheme (Grimont and Weil, 2007) at the laboratory of Public Health Agency of Turkey in Ankara. Isolates were maintained at 80 °C in 15% (vol/vol) glycerol-brain heart infusion (BHI) medium.

### 2.2. Genome sequencing

Genomic DNA from each strain was extracted from overnight cultures using the DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA). Libraries were constructed using 1 ng of genomic DNA and the Nextera XT DNA library preparation kit (Illumina, San Diego, CA). The genomes were sequenced with the NextSeq 500/550 high-output kit version 2 (300 cycles) on a NextSeq 500 instrument (Illumina, San Diego, CA), following the manufacturer's instruction. The sequencing experiments were performed as a part of an FDA Center for Food Safety and Applied Nutrition's surveillance project. The sequencing was a part of GenomeTrakr Project: Turkey (BioProject 266622) with individual accession numbers provided in the Supplementary Table 1.

### 2.3. Genome assembly

Read quality control and trimming, genome *de novo* assembly, and SNP analysis proceeded essentially as described in (Stasiewicz et al., 2015). Briefly, raw read quality was assessed with FastQC (v0.10.1 [http://www.bioinformatics.babraham.ac.uk/projects/fastqc/]). Illumina adapter sequences and low-quality sequence were trimmed using Trimmomatic (v0.32) (Bolger et al., 2014) default settings. Trimmed, paired reads were *de novo* assembled using SPAdes (v3.8.2) (Bankevich et al., 2012) with the suggested k-mer set for prokaryotic assembly: 21, 33, 55, 77, 99, and 127 bp. After trimming, any contig < 500 bp in length or with a < 1× average k-mer coverage was removed.

### 2.4. SNP analysis

The Cortex variation assembler (v.10.5.21) (Iqbal et al., 2012) was used to detect SNPs, indels, and complex variants using a reference-based workflow and the closely-related *Salmonella* Infantis strain SINFA reference genome (GCA_000953495.1). Here, a diversity set of 243 additional *Salmonella* Infantis genomes was added to the analysis (see Supplementary Table 1). These were all the *Salmonella* Infantis present in the GenomeTrakr: *Salmonella* project (Bioproject 183844) as of 5/16/2016. Only SNPs were retained for phylogenetic analysis using RAxML v8.2.4 (Stamatakis, 2014) using a GTR model of nucleotide substitution and 100 bootstrap replications to generate support values. After trimming, any contig < 500 bp in length or with a < 1× average k-mer coverage was removed. Phylogenetic trees were drawn using


**Fig. 1.** Phylogenetic tree based on whole-genome SNP analysis of the 21 *Salmonella* Infantis isolates from Turkey and 243 comparison isolates. All Turkish isolates form a single, well-supported phylogenetic clade, presented in expanded view, with bootstrap support values given at nodes. Full SNP tree with all 264 taxa individually labeled is presented in Supplemental Fig. 1.

MEGA v7.0.14 (Tamura et al., 2013).

### 2.5. Detection of genes encoding antimicrobial resistance and virulence factors

The genomes were screened for the antimicrobial resistance genes (*aacG*, *aadA*, *aph3″la*, *cmlA*, *dfrA5*, *dfrA8*, *qnrS*, *sulIII*, *sulI*, *blaTEM-1D*, *tetA* and *tetR*) using SRST2 (Inouye et al., 2014); this software allows the detection of genes of interest directly from short reads and with higher sensitivity than assembly-based approaches. For this analysis we used a copy of the ARG-ANNOT antimicrobial resistance genes database (Gupta et al., 2014) downloaded on 11/17/2016 and mapped only the first 400,000 quality trimmed reads for each isolate. Sensitivity and specificity values were computed according to the protocol of Altman and Bland (1994) to compare the AMR profiles found by PCR and WGS.

Plasmid-encoded virulence and bacteriocin-resistance genes (*vagC*, *ccdA*, *ccdB*, *pilS*, *tcpT*, *nikB*, *trbA*, *trbB*, *mchE*, and *cvaB*) were detected after annotation of plasmid genome with Bacterial Annotation System (BASys) (Van Domselaar et al., 2005).

To generate a comparison ARG content data for *in silico* results, ARG profiling by PCR was performed on the phenotypically resistant isolates. 21 antimicrobial resistance coding genes (*blaTEM-1*, *blaPSE-1*, *blaCMY-2*, *ampC*, *cat1*, *cat2*, *flo*, *cmlA*, *aadA1*, *aadA2*, *strA*, *strB*, *aacC2*, *aphA1-Iab*, *dhfrI*, *dhfrXII*, *sulI*, *sulII*, *tetA*, *tetB*, *tetG*) were amplified to determine genetic variation of AR (Soyer et al., 2013). The genes and the primers that were studied were given in Supplementary Table 2. Salmonella DNA was isolated prior to genotypic AR profiles analysis by DNA4U® Bacterial Genomic DNA Isolation Kit (Nanobiz, Ankara, Turkey).

### 2.6. Detection of plasmids

Plasmids were identified by plasmidSPAdes (Antipov et al., 2016), which uses the read coverage of generated contigs to distinguish between chromosomal and plasmid DNA. Plasmid contigs were verified using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The assemblies were annotated by submission to the Bacterial Annotation System (BASys; http://wishart.biology.ualberta.ca/basys/cgi/submit.pl).

Plasmids with highly similar sequences were identified and analyzed using the BLAST pairwise alignment to construct neighbor joining trees to identify the plasmids with the highest degree of similarity to the plasmids in the study. Additionally, plasmids with > 50% query coverage were imported into the Mauve (ver. 2.0) program to conduct multiple alignments to compare the distribution of determinants, such as those potentially associated with antimicrobial resistance, virulence, and transfer as identified in the sequenced plasmids with those in the GenBank database.

## 3. Results and discussion

### 3.1. Comparison of Turkish Salmonella Infantis isolates to international isolates

The SNP analysis revealed that the isolates were clustered by geography and by host. The Turkish *Salmonella* Infantis isolates, that were from chicken meat sources, were all clustered together in one well-supported clade (Fig. 1). It is interesting to observe the geographical distribution of the *S*. Infantis isolates (Supplemental Fig. 1). The clusters most related to Turkish isolates were from Egypt and Israel, whereas the clusters least related were from mostly the U.S., China, and Mexico. Therefore, these data suggest a more recent common ancestor for the isolates from Egypt, Israel and Turkey. Also, the isolates, located in the second largest cluster were all from chicken sources, indicating that the clusters were also dependent on host.

Analysis of 243 *Salmonella* Infantis isolates, obtained from NCBI database, shows that most of them (166/208) were from U.S., while only 20% of them were from other geographical locations such as Brazil (1), Canada (3), China (2), Egypt (2), India (1), Israel (1), Mexico (9), Thailand (1) and Turkey (22) (Supplementary Table 3). This outcome is not likely a result of higher prevalence but rather increased representation of U.S. isolates overall in the U.S.-led genome sequencing efforts. But it emphasizes that work in this area should be enhanced and supported with data from other continents and countries.

Also, it is observed that the isolates at NCBI database were mostly frequently (34%) obtained from poultry and poultry-associated environment, followed by porcine (14%), and unknown environmental sources (11%). This supports the frequent identification of poultry-associated *S*. Infantis worldwide (Jackson et al., 2013; Hindermann et al., 2017; Nógrády et al., 2007). There was only one human-related *S*. Infantis isolate, from New York, USA, and the most closely related clusters contained isolates from chicken sources (Supplementary Fig. 1).

### 3.2. ARGs found within whole genome sequence data and comparison of them to PCR-based identification and phenotypic resistance data

Whole genome sequence analysis for detection of ARGs identified 13 genes across 7 classes (Table 1). Comparing those results to previously collected PCR-based results, for the subset of those genes found in both studies (*aadA*, *aphA*, *tetA*, *sul1*, *str* (Acar et al., 2017)), WGS methods were 100% sensitive in detecting genes found in PCR-screen. In addition to these genes, by WGS we found additional genes that were not tested for by PCR-based methods in our previous studies: *aac6*, *tetR*, *dhfrV*, *dfrA14* and *qnrS1*.

Considering phenotypes, WGS methods identified 6 distinct antimicrobial resistance profiles. In comparison, phenotypic work identified 9 distinct profiles. Overall, 94% similarity was found for phenotypic AMR profiles between PCR-based and WGS-based results. The differences were in such as; MET-S1-688 where *tetR* gene was identified by WGS but not by PCR, nor was the strain phenotypically resistant to tetracycline.

Bioinformatics identification of the *aac6* gene potentially conflicts with phenotypic data. The *aac6* gene, GCN5-related *N*-acetyltransferase, is related to gentamicin, tobramycin, or amikacin resistance in literature (Stogios et al., 2016). In our phenotypic data there is not support for resistance to gentamicin or amikacin. The disk diffusion diameters of the *S*. Infantis isolates which harbored the *aac6* gene were checked to see if those isolates were resistant or intermediately susceptible - results were susceptible for all. The *aac6* gene was not related to gentamicin resistance in our *S*. Infantis isolates since disk diameters of *aac6* gene detected isolates were high as 19–21 cm (while resistance diameter of gentamicin is 12–15 cm). Similar with amikacin, with 14–17 cm of resistance diameter and disk diffusion result of 19–21 cm (data not shown). Still, the *aac6* gene may still be related to tobramycin resistance, but resistance to that compound was not studied here. Additionally, the *aac6* gene was also detected in other isolates with phenotypic streptomycin resistance (Del Campo et al., 2000; Cavaco et al., 2007) and streptomycin resistance in our *S*. Infantis isolates was correlated with *aac6* presence; hence the *aac6* gene may contribute to streptomycin resistance.

To the best of our knowledge, the three beta-lactam resistance gene variants $bla_{\text{TEM-70}}$, $bla_{\text{TEM-148}}$ and $bla_{\text{TEM-198}}$ have not previously been reported in *Salmonella* Infantis. The beta-lactam resistance gene, $bla_{\text{TEM-70}}$, which is found on MET-S1 56, was originally found in an *Escherichia coli* isolated in Russia. Similarly, $bla_{\text{TEM-148}}$ (from MET-S1 669) was originally found in an *Escherichia coli* isolated in Portugal. Finally, $bla_{\text{TEM-198}}$ (from MET-S1 50) was originally found in a *Klebsiella pneumoniae* isolated in Japan (https://www.ncbi.nlm.nih.gov/nuccore). The three beta lactamase genes, $bla_{TEM\text{-}70}$, $bla_{TEM\text{-}148}$ and $bla_{TEM\text{-}198}$, all diverged from $bla_{\text{TEM-1}}$ by one single amino acid substitution, arginine to glutamine at site 204, threonine to lysine at site 189 and threonine to isoleucine at site 271, respectively.

### 3.3. S. Infantis plasmids

In our study, plasmids were detected among 91.3% of *S*. Infantis (21/23) isolates, with the sizes 10 kb to 280 kb. 47.6% of the found plasmids (10/21) were sized > 200 kb, with 33.3% of them (7/21) longer than 250 kb (Table 2).

There is evidence that there is an increase of extended-spectrum-beta-lactamase (ESBL)-producing *S*. Infantis all over the world, especially U.S. and Europe. Isolates were linked to the pESI (plasmid for emerging *S*. Infantis) or pESI-like mega-plasmids (approximately 300 kb) with the incompatibility group IncX1 (Tate et al., 2017). The plasmids in this study, which were longer than 200 kb, were highly genetically similar (130 kb region sharing 100% identity and 100% query coverage) (Fig. 2) with the plasmids of *S*. Infantis N55391, FSIS1502916, and FSIS1502169 from Tate et al., 2017 with plasmid sizes 316 to 323 kb Those three comparison *S*. Infantis were isolated from chicken sources in recent years, 2014–2015, from different U.S. regions, Tennessee, New Jersey and North Carolina. For comparison, N55391 had an antimicrobial resistance profile of ampicillin, ceftiofur, ceftriaxone, chloramphenicol, nalidixic acid, cefotaxime, aztreonam and tetracycline. Only three isolates in this study had $bla_{\text{TEM}}$ resistance, and had genes: $bla_{\text{TEM-70}}$ found in isolate MET-S1-56, $bla_{\text{TEM-148}}$ found in isolate MET-S1-669 and $bla_{\text{TEM-198}}$ found in isolate MET-S1-50 (Table 1).

The plasmids larger than 250 kb were found to have four large contigs which were 70 kb, 60 kb, 45 kb and 35 kb (Fig. 2). The former two contigs were mostly found together sizing nearly 130 kb (Site 1) and the latter two contigs were also usually as a group with the size of approximately 75 to 110 kb (Site 2) (Table 2). Site 1 had the streptomycin resistance gene (*str*), virulence genes (*vagC*, *ccdA-ccdB*), conjugation genes (nik genes, tra genes, etc) and also colicin (*cvaB*) and microcin (*mchE*) secretion genes. 60% of the plasmids had a Site 1 region with the sizes ~120 to ~140 kb and 50% of the plasmids had a Site 2 region with the sizes of ~75 to ~110 kb. The antimicrobial resistance genes (*aadA*, *sulI*, *tetA*, *tetR*) were found on transposons (Tn552 and Tn501) together with mercury resistance genes (mer) on Site 2. The *mer* gene is known as one of the backbone gene of plasmids, especially with the transposon Tn21 family. Similar to our data, the Italian (Franco et al., 2015) and U.S. (Tate et al., 2017) *S*. Infantis plasmids also contained the *mer* gene together with antimicrobial resistance genes.

According to the WGS data, MET-S1-668 and MET-S1-672 had no plasmids while their phenotypic antimicrobial resistance patterns (SSfN and KSTSfN, respectively) and genetic antimicrobial resistance types (SRST2 types of AR3 and AR2, respectively) were very common among other plasmid harboring isolates (Table 1). We suspect this result is an artifact of the bioinformatic analysis. The algorithm used to detect plasmids in this study was *plasmidSPAdes* (Antipov et al., 2016), which is completely automated, and independent of reference sequences.

**Table 1**
Comparison of antimicrobial genes detected by phenotypic and in-silico methods.

| SRR code | METU code | Phenotypic resistance profile[a] | SRST2[b] type | Aminoglycoside | | Sulfonamide | | Tetracycline | | Trimethoprim | | Beta-lactam | | Chloramphenicol | | Quinolones | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | SRST2 | Lab data | SRST2 | Lab data | SRST2 | Lab data | SRST2 | Lab data | SRST2 | Lab data | SRST2 | Lab data | SRST2 | Lab data |
| 1916126 | MET-S1-050 | KSTAmpSfN | AR4 | *aac6*[c], *aadA1*, *aph3″Ia* | *aphA1*, *aadA1* | *sulI* | *sulI* | *tetA*, *tetR*[c] | *tetA* | *dfrA14* | *N/A*[c] | *blaTEM-198* | *blaTEM-1* | – | – | *qnr-S1* | *N/A*[c] |
| 1916111 | MET-S1-056 | KSTAmpKfSfSxtCN | AR1 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*, *aadA1* | *sulI*, *sulIII* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | *blaTEM-70* | *blaTEM-1* | *cmlA1* | *cmlA* | – | *N/A* |
| 1916122 | MET-S1-088 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1916080 | MET-S1-092 | STSfN | AR3 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1916073 | MET-S1-142 | STSfN | AR3 | *aac6*, *aadA1* | *aadA1*, *strA* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1916101 | MET-S1-150 | STSfN | AR3 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1745554 | MET-S1-329 | STSfN | AR3 | *aac6*, *aadA1* | *aadA1*, *strA* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1745619 | MET-S1-345 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1745538 | MET-S1-351 | STSfN | AR3 | *aac6*, *aadA1* | *aadA1*, *strA* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1810872 | MET-S1-492 | STN | AR3 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1810850 | MET-S1-498 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1810851 | MET-S1-510 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1812878 | MET-S1-597 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1812857 | MET-S1-606 | STSfN | AR3 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1812829 | MET-S1-668 | SSfN | AR3 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | – | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1916099 | MET-S1-669 | SAmpKfN | AR5 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | – | – | *N/A* | *blaTEM-148* | *blaTEM-1* | – | – | – | *N/A* |
| 1916072 | MET-S1-671 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1916109 | MET-S1-672 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1916087 | MET-S1-673 | TN | AR6 | *aac6*, *aadA1* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | – | *N/A* | – | – | – | – | – | *N/A* |
| 1812814 | MET-S1-676 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | – | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1812796 | MET-S1-686 | KSTSfN | AR2 | *aac6*, *aadA1*, *aph3″Ia* | *aphA1*,*aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |
| 1812815 | MET-S1-689 | TSfN | AR6 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | *tetA* | – | *N/A* | – | – | – | – | – | *N/A* |
| 1916110 | MET-S1-699 | STSfN | AR3 | *aac6*, *aadA1* | *aadA1* | *sulI* | *sulI* | *tetA*, *tetR* | – | *dfrA14* | *N/A* | – | – | – | – | – | *N/A* |

[a] The abbreviations for the antimicrobial resistance patterns; Amp: ampicillin, K: kanamycin, Kf: cephalothin, N: nalidixic acid, S: streptomycin, Sf: sulfonamide, Sxt: trimethoprim-sulphamethoxazole, T: tetracycline.
[b] SRST2: A program; Short Read Sequence Typing for Bacterial Pathogens (Inouye et al., 2014).
[c] *aac6* &, *tetR*, *dfrA14* and *qnr-S1* genes were not analyzed in wet-lab PCR studies.

**Table 2**
The distribution of Site 1[a] and Site 2[b] in the plasmids of Turkish *Salmonella* Infantis.

| METU code | Total plasmid size (kb) | Virulence associated Site 1 size (kb) | Antimicrobial resistance associated Site 2 size (kb) |
|---|---|---|---|
| MET-S1-050 | 216 | 132 | 77 |
| MET-S1-056 | 158 | 124 | – |
| MET-S1-088 | 219 | 96 | 105 |
| MET-S1-092 | 15 | – | 8 |
| MET-S1-142 | 165 | 119 | 18 |
| MET-S1-150 | 165 | 144 | 14 |
| MET-S1-329 | 255 | 132 | 110 |
| MET-S1-345 | 245 | 132 | 105 |
| MET-S1-351 | 36 | – | 36 |
| MET-S1-492 | 286 | 131 | 77 |
| MET-S1-498 | 176 | 132 | 40 |
| MET-S1-510 | 283 | 131 | 105 |
| MET-S1-597 | 248 | 133 | 77 |
| MET-S1-606 | 282 | 135 | 64 |
| MET-S1-668 | 0 | – | – |
| MET-S1-669 | 64 | 64 | – |
| MET-S1-671 | 84 | 84 | – |
| MET-S1-672 | 0 | – | – |
| MET-S1-673 | 13 | – | 12 |
| MET-S1-676 | 59 | – | – |
| MET-S1-686 | 248 | 99 | 102 |
| MET-S1-689 | 219 | 131 | 88 |
| MET-S1-699 | 175 | 64 | 77 |

[a] Site 1 genes of interest; vagC: Virulence-associated protein; ccdA: protein CcdA; ccdB: Cytotoxic protein CcdB [H]; mchE: Microcin H47 secretion protein mchE [H]; cvaB: Colicin V secretion/processing ATP-binding protein CvaB [H]; str: Streptomycin 3″-kinase [H];

[b] Site 2 genes of interest: mer: Mercury resistance protein; aadA: Streptomycin 3″-adenylyltransferase [H]; sulI: Dihydropteroate synthase type-1 [H]; tetA: Tetracycline resistance protein, class A; tetR: Tetracycline repressor protein class A from transposon 1721; tnpA: Transposase for transposon Tn501; tcpT: Toxin coregulated pilus biosynthesis protein T [H]; tra: Transfer operon.

Recent work shows that *plasmidSPAdes* fails to correctly rebuild all plasmid structures from short-read WGS datasets, and it may identify plasmid-derived sequence without structural accuracy (Orlek et al., 2017). The contigs obtained by *plasmidSPAdes* were checked with NCBI BlastN to remove chromosomal DNA. Because it is known that the precision of *plasmidSPAdes* is 0.75, indicating that the software gives many false positives (Arredondo-Alonso et al., 2017). Since prediction of large plasmids (> 50kbs) with repeated sequences is problematic in many plasmid detection algorithms; contigs with low copy number may have been discarded in this study due to the algorithm used.

### 3.4. Antimicrobial resistance genes on plasmids

*aadA1*, *tetA*, and *sulI* genes were observed in *Salmonella* Infantis WGS data with prevalence of 100%, 83% and 100% respectively. But their abundance in the computationally identified plasmid data is lower, 70% for *aadA1*, 48% for *tetA*, and 65% for *sulI*. This result suggests either that (i) the genes are not located on plasmids or that (ii) future work is need to identify complete plasmid sequences.

When resistance genes were identified on plasmids, they were mainly located within transposons and insertion elements. *aadA1* and *sulI* genes were found together on transposon Tn552, a beta-lactamase encoding transposon (Rowland and Dyke, 1990). We did not observe beta lactamase genes among the three plasmids possibly due to the loss of some parts during plasmid detection. *tetA* and *tetR* genes were located together on transposon Tn501, with mercury resistance gene cassettes. The insertion sequences found, ISR1 and ISRM3, harbored *dhfrV* and *str* resistance genes, respectively (Fig. 3).

### 3.5. Other important genes carried on plasmids include virulence- and bacteriocin-related genes

The emergence of a strain is not only dependent on antimicrobial resistance but also on different factors such as virulence, conjugation ability, and other characteristics like bacteriocin secretion. The genes encoding these characteristics were screened, and the notable ones were listed as: *vagC* (virulence-associated protein encoding gene), *ccdA* (cytotoxic CcdA protein encoding gene), *ccdB* (cytotoxic CcdA protein encoding gene), *tcpT* (toxin coregulated pilus biosynthesis gene), *mchE* (microcin H47 secretion protein encoding gene) and *cvaB* (colicin V secretion/processing ATP-binding protein encoding gene) (Table 3). Colicin and microcin secretion related genes were all found together on 75% of the Turkish *S*. Infantis plasmids. TnpA gene (Fig. 3), which is associated with transposon Tn501 (a common transposon found on plasmids and functions in AMG transmission), was observed in 55% of them. Also, 60% of the plasmids, had the *ccdA-ccdB* gene complex and *vagC* gene.

Virulence and resistance are known to evolve together (Beceiro et al., 2013), negatively affecting food safety and human health. *vagC* (virulence associated protein encoding gene) was observed very commonly in plasmids, especially the IncF plasmids. An example is pEK499, which is an *Escherichia coli* plasmid, harboring the genes encoding resistance to β-lactamases ($bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{OXA-1}}$), aminoglycosides (*aac6-Ib-cr*), chloramphenicol (*catB4*), tetracycline [*tet* (*A*)], streptomycin (*aadA5*), and sulfonamide (*sulI*) (Woodford et al., 2009). It has two replicas of the *vagD-vagC* virulence-associated system and *ccdA-ccdB* systems. They are known to be involved in plasmid maintenance by post segregation killing processes. Plasmid stability was found be maintained by *vagD-vagC* virulence-associated system by modulating cell division in *Salmonella* Dublin isolates (Pullinger and Lax, 1992).

Type II toxin-antitoxin systems (TAs) are known to affect the ecology of *Salmonella enterica* isolates. They directly influence adaptability and persistence of the most prevalent *Salmonella* serovars (Di Cesare et al., 2016). Furthermore, the TAs *ccdAB* (coupled cell division locus), which is involved in plasmid maintenance, were frequently identified in this current study. The gene *ccdAB* was previously found in pSLT, the virulence plasmid of *S*. Typhimurium (Lobato-Márquez et al., 2015) and the complex inhibits DNA gyrase activity. Interestingly, the *ccdAB* and *vagCD* gene complexes have been found in the plasmids of *Salmonella* serovars of Typhimurium, Enteritidis, Heidelberg, Dublin, Pullorum, Choleraesuis and Typhi (Chen et al., 2007; Chu et al., 2008; Dhanani et al., 2015; Feng et al., 2012) but not in the serovar Infantis. Thus, this study shows the possible threat of *S*. Infantis and suggests a contributing factor to why *S*. Infantis has emerged from the 2000s to 2014, becoming the fourth most common *Salmonella* serovar in humans in EU (EFSA&ECDC, 2015).

Conjugation related genes such as *pilS*, f pilus, *nikA*, *nikB*, *trbA*, and *trbB* were commonly (10/21) observed in plasmids of Turkish *S*. Infantis isolates. Conjugation in *Salmonella* Infantis mega-plasmid (pESI, approximately 280 kb) was previously studied with gut microbiota of warm-blooded hosts (Aviv et al., 2016) and it was found that pESI can be horizontally transferred to the gut microbiota during *Salmonella* infection. So it should be emphasized that conjugation related genes such as pilus genes (f pilus and *pilS*) and *nikAB* gene complexes (nickel binding periplasmic protein gene) are gaining importance in prevalence of antimicrobial resistant and virulent *Salmonella* serovars.

Colicin and microcin are known as next generation bacteriocins and toxic bacterial exoproteins (Gillor et al., 2004), where they can kill susceptible cells by disrupting their cell membrane. Plasmid- or chromosome-encoded antibiotics are produced by Enterobacteriaceae family, mostly *Escherichia coli* strains and they were found to inhibit the growth of several pathogenic *E. coli*, *Salmonella* and *Shigella* strains (Rintoul et al., 2001). Thus, some patented microcin and colicin applications have been developed for poultry and cattle industry.

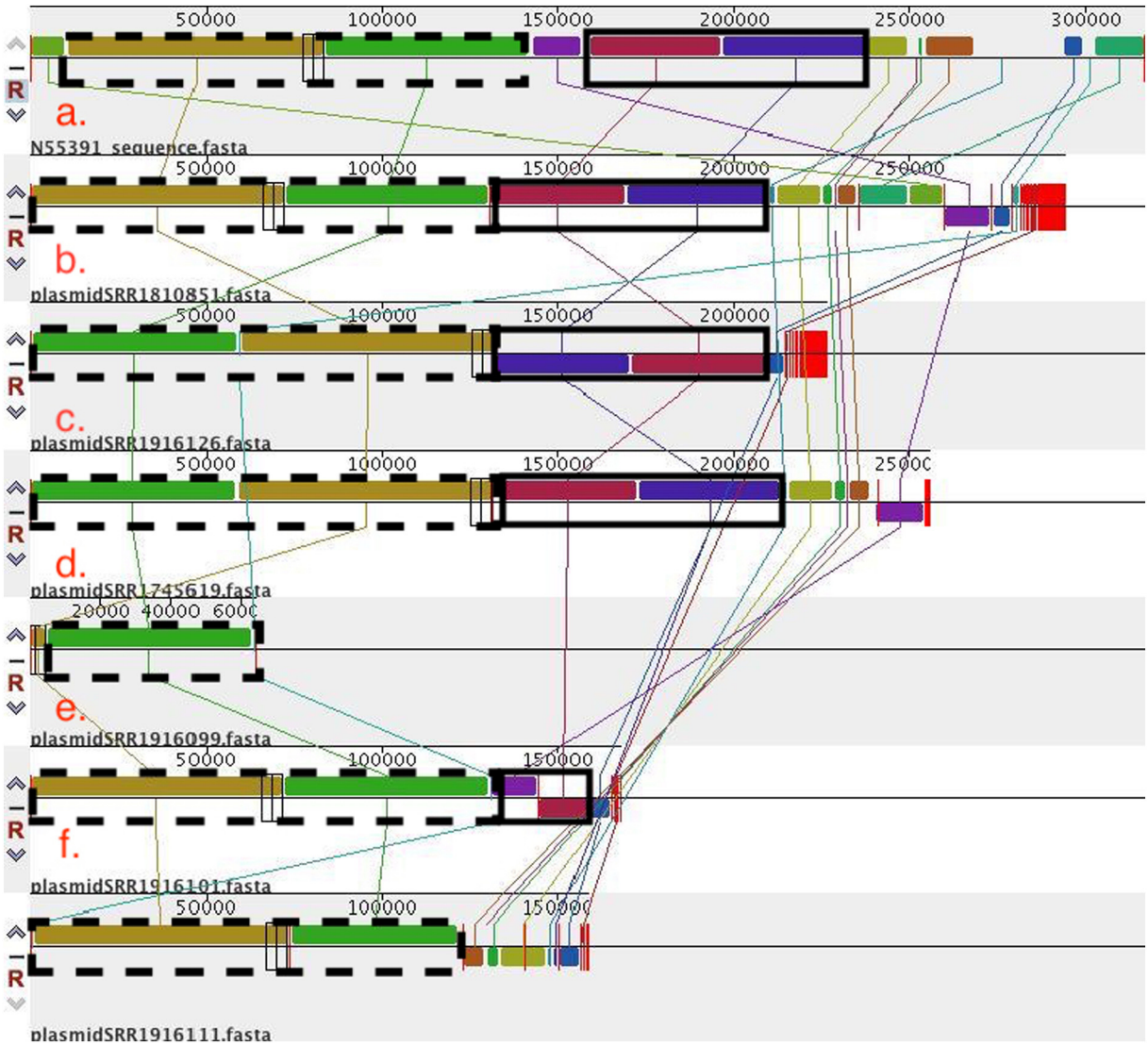


**Fig. 2.** The distribution of the contigs in Turkish S. Infantis plasmids with a comparison of plasmid N55391 (Tate et al., 2017). The figure emphasizes that there are two parts; 130 kb (yellow and green) and 90 kb (red and blue), which are dispersed in different alignments in plasmids. a. plasmid of S. Infantis N55391, b. plasmid of MET-S1-510 (283 kb), c. plasmid of MET-S1-50 (216 kb), d. Plasmid of MET-S1-345 (255 kb), e. plasmid of MET-S1-669 (64 kb), f. plasmid of MET-S1-150 (165 kb) and g. plasmid of MET-S1-56 (158 kb) (Site 1 and Site 2 are identified in dashed and regular rectangles respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Microcin and colicin secretion protein encoding genes were found together in Turkish *Salmonella* Infantis isolates. This result was predicted because microcin H47 was shown to be secreted by a three-component ATP-binding cassette exporter, strongly associated with a colicin V exporter (Azpiroz et al., 2001). *cvaB*, which is a colicin V secretion protein, is associated with an *E. coli* plasmid. But recently it was found in a *S*. Kentucky that was isolated from broiler processing facilities in U.S. The ColV plasmid increased the isolates colonization and fitness capabilities (Johnson et al., 2010). Also in this recent study we found *cvaB* gene in 65% of all plasmids found in Turkish *Salmonella* Infantis isolates, showing the potential for horizontal gene transfer of the gene from *Escherichia coli* to *Salmonella* Infantis in a poultry environment. *mchE* is a microcin H47 secretion protein encoding gene, and is mostly associated Enterobacteriaceae family (*E. coli* and *Yersinia enterocolitica*) (Azpiroz and Laviña, 2007). In literature, it was found in one of the *Salmonella* isolates in NCBI database, *Salmonella enterica* subsp. *indica* plasmid, pSARC14-41, while it is mostly studied to be used as a probiotic to inhibit *Salmonella* (Palmer et al., 2017). These two commonly found genes suggest additional potential human health hazard of AMR *S*. Infantis infection due to plasmid-borne resistance.

## 4. Conclusion

This study demonstrated that the plasmids of Turkish S. Infantis isolates had similar antimicrobial resistance and plasmid presence characteristics with Italian, Hungarian and U.S. poultry-related *S*.

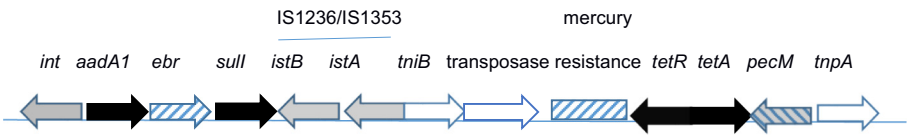


**Fig. 3.** Physical structure of integron and transposon associations of *S*. Infantis isolates. Black arrows, antimicrobial resistance genes; white arrows, transposases: Tn552 (transposase) and Tn501 (*tnpA*) respectively; *pecM*, drug transporter protein, mostly. IS, insertion sequence.

**Table 3**
Annotation results of plasmids of Turkish isolates showing important genes of interest[a].

| METU code | Plasmid size (Kb) | Resistance genes | Virulence genes of interest | Conjugation genes of interest | Other genes |
|---|---|---|---|---|---|
| MET-S1-351 | 36 | *aadA*, *sulI*, *tetA*, *tetR* | *tcpT* | | *tnpA* |
| MET-S1-345 | 255 | *str*, *aadA* | *vagC* | *nikA*, *nikB*, *trbA*, *trbB* | *mchE*, *cvaB* |
| MET-S1-329 | 245 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV* | *vagC*, *ccdA*, *ccdB*, *tcpT* | *nikA* | *mchE*, *cvaB*, *tnpA* |
| MET-S1-498 | 176 | *str*, *aphA1* | *vagC*, *ccdA*, *ccdB* | | *mchE*, *cvaB* |
| MET-S1-510 | 283 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV*, *aphA1* | *vagC*, *ccdA*, *ccdB*, *tcpT* | | *mchE*, *cvaB*, *tnpA* |
| MET-S1-492 | 286 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV* | *ccdA*, *ccdB* | *nikA*, *nikB*, *trbA*, *trbB* | *mchE*, *cvaB*, *tnpA* |
| MET-S1-686 | 248 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV*, *aphA1* | *vagC*, *ccdA*, *ccdB*, *tcpT* | *trbA*, *trbB*, *f pilus*, *pilS* | *mchE*, *cvaB*, *tnpA* |
| MET-S1-676 | 59 | – | – | – | – |
| MET-S1-689 | 219 | *aadA*, *sulI*, *tetA*, *tetR* | *vagC*, *ccdA*, *ccdB*, *tcpT* | *f pilus* | *mchE*, *cvaB*, *tnpA* |
| MET-S1-668 | ND | – | – | – | – |
| MET-S1-606 | 282 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV* | *vagC*, *ccdA*, *ccdB*, *tcpT* | *pilS*, *f pilus* | *mchE*, *cvaB*, *tnpA* |
| MET-S1-597 | 248 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV*, *aphA1* | *vagC*, *ccdA*, *ccdB*, *tcpT* | *nikA*, *nikB*, *pilS*, *f pilus* | *mchE*, *cvaB*, *tnpA* |
| MET-S1-671 | 84 | *str* | – | – | *mchE*, *cvaB* |
| MET-S1-142 | 165 | *str*, *aadA*, *sulI*, *dhfrV* | *ccdB* | – | *mchE*, *cvaB* |
| MET-S1-92 | 15 | *aadA*, *sulI*, *dhfrV* | – | – | – |
| MET-S1-673 | 13 | *aadA*, *sulI* | – | – | – |
| MET-S1-669 | 64 | *str* | – | – | – |
| MET-S1-150 | 165 | *str*, *aadA*, *sulI*, *dhfrV* | *vagC*, *ccdA*, *ccdB* | *nikA*, *nikB* | *mchE*, *cvaB* |
| MET-S1-672 | ND | ND[b] | – | – | – |
| MET-S1-699 | 175 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV* | *tcpT* | *trbA*, *trbB*, *f pilus* | *tnpA* |
| MET-S1-56 | 158 | *str*, *dhfrV* | *vagC*, *ccdA*, *ccdB* | – | *mchE*, *cvaB* |
| MET-S1-88 | 219 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV*, *aphA1* | *vagC*, *ccdA*, *ccdB*, *tcpT* | *trbA* | *mchE*, *cvaB*, *tnpA* |
| MET-S1-50 | 216 | *str*, *aadA*, *sulI*, *tetA*, *tetR*, *dhfrV* | *vagC*, *ccdA*, *ccdB*, *tcpT* | *nikA*, *nikB*, *trbA*, *trbB*, *pilS* | *mchE*, *cvaB*, *tnpA* |

[a] Genes of interest: *aadA*: Streptomycin 3″-adenylyltransferase [H]; *aphA1*: Aminoglycoside 3′-phosphotransferase [H]; *ccdA*: protein CcdA; *ccdB*: Cytotoxic protein CcdB [H]; *cvaB*: Colicin V secretion/processing ATP-binding protein CvaB [H]; *dhfrV*: Dihydrofolate reductase type 5 [H]; *mchE*: Microcin H47 secretion protein mchE [H]; *nikB*: Plasmid Conjugation System; *sulI*: Dihydropteroate synthase type-1 [H]; *pilS*: Conjugative Transfer Outer Membrane Protein PilS, TypeIV Prepilin; *str*: Streptomycin 3″-kinase [H]; *tetA*: Tetracycline resistance protein, class A; *tetR*: Tetracycline repressor protein class A from transposon 1721; *tcpT*: Toxin coregulated pilus biosynthesis protein T [H]; *tnpA*: Transposase for transposon Tn501; *trbA/B*: Conjugal Transfer Protein; *vagC*: Virulence-associated protein.
[b] ND: not detected.

Infantis (Franco et al., 2015; Nógrády et al., 2012; Tate et al., 2017). A nearly 300 kb plasmid with antimicrobial resistance genes, mercury resistance genes, insertion elements, and transposons had been previously identified in these studies and was found in the study reported here. Interestingly, our findings show microcin and colicin secretion capability of plasmids, which may play a part in the emergence of *S.* Infantis among other serovars and species. Also, type II toxin-antitoxin systems associated genes such as *ccdA-ccdB* and *vagC* were first identified in *S.* Infantis isolates in this study. All these present the genomic evidence of MDR *S.* Infantis isolates with virulence ability due to their plasmids.

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