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Salmonella Enteritidis Isolate Harboring Multiple Efflux Pumps and Pathogenicity Factors, Shows Absence of O Antigen Polymerase Gene

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Keywords: Salmonella Enteritidis, omphalitis, wzy deletion, epidemiology, pathogenicity factors, MGE, metal tolerance

BACKGROUND

Salmonella enterica is one of the most important causes of gastrointestinal infection in humans, being the great majority of infections related to the consumption of poultry meat and eggs (Foley and Lynne, 2008; EFSA/ECDC, 2015).

In animals, infections caused by serotype Enteritidis are rarely responsible for severe disease with animals frequently becoming asymptomatic carriers, except in the case of young chicks and poults, where outbreaks exhibiting clinical disease are often accompanied by high mortality rates (Foley et al., 2008, 2013). Indeed, S. enterica subsp. enterica serovar Enteritidis (S. Enteritidis) has been responsible for severe disease in industrial poultry farming facilities worldwide, posing a potential hazard for public health (Lutful Kabir, 2010).

In order to be infectious, Salmonella needs to adapt to different niches and conditions, where virulence and heavy-metal-tolerance factors play an important role, through co-selection events and the formation of pathogenicity islands, respectively (Hensel, 2004; Medardus et al., 2014). Furthermore, antibiotic resistance determinants can also facilitate their survival, with ubiquitous chromosomally encoded efflux mechanisms, playing an important role in both intrinsic, and acquired multidrug resistance. Other resistance mechanisms, such as changes in the membrane permeability, enzymatic modification, and target alterations may increase the levels of bacterial resistance, contributing to the success of the infection (Poole, 2004; Delmar et al., 2014; Li et al., 2015).

Both antibiotic susceptibility determination and serotyping constitute very useful tools for the epidemiologic classification of S. enterica isolates. Indeed, in S. enterica, the resistance rates fluctuate according to the serotype and with the antibiotic (Clemente et al., 2015). Classically, serotyping is based on the antigenic reactivity of lipopolysaccharide (O antigen) and flagellar proteins (H antigen), followed by a designation using names or formulas (Grimont and Weill, 2007). In this study, we aimed to analyze the genome of a S. Enteritidis isolate responsible for omphalitis in chicks,
exploring the molecular features associated with antibiotic resistance and pathogenicity, as well as the ability to spread the respective determinants.

**METHODS**

**Bacterial Isolate, Antibiotic Susceptibility Testing, and Serotyping**

The isolate (LV60) was recovered from a sample collected from the yolk sac of a chick with omphalitis, under the scope of the “Salmonella National Control Programme in food-producing animals and food of animal origin for bacteriological diagnosis, serotype identification and antibiotic susceptibility testing.” The guidelines of the Commission Decision (CD), 2007/407/EC were followed. LV60 was tested for its antimicrobial resistance through the determination of minimum inhibitory concentrations (MICs) using the agar dilution method, as previously described (Clemente et al., 2013) and according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/). Briefly, a panel of 11 antibiotic compounds was tested in a 2-fold concentration series over the following ranges: ampicillin and tetracycline (0.5–64 µg/mL), gentamicin and trimethoprim (0.25–32 µg/mL), ciprofloxacin (0.008–8 µg/mL), cefotaxime (0.06–8 µg/mL), nalidixic acid and streptomycin (2–512 µg/mL), chloramphenicol (2–256 µg/mL), florfenicol (1–128 µg/mL) and sulphamethoxazole (8–1024 µg/mL). The epidemiological cutoff values recommended by EUCAST to *Salmonella* spp. were

| Reference Position | Reference | Allele | Gene (Product) | Amino acid change | Coverage |
|--------------------|-----------|--------|----------------|-------------------|----------|
| 40158              | C         | T      | SEN_RS00180 (aryl sulfatase) | Pro92Ser | 155      |
| 55278              | C         | A      | ileS (isoleucine-tRNA ligase) | Ala557Glu | 144      |
| 90379              | G         | A      | SEN_RS00415 (hypothetical protein) | Ala96Thr | 127      |
| 156264             | G         | A      | SEN_RS00685 (peptidase M23) | Gly999Asp | 123      |
| 353437             | T         | C      | SEN_RS01625 (isopropylylmalate isomerase) | Val454Ala | 119      |
| 357149             | A         | T      | SEN_RS01625 (hypothetical protein) | Leu1Met | 177      |
| 401018             | C         | A      | prpE (acetyl-CoA synthetase) | Arg9Ser | 132      |
| 411602             | T         | G      | SEN_RS01845 (hypothetical protein) | Trp209Gly | 58       |
| 561577             | T         | C      | SEN_RS20580 (MFS transporter) | Ser333Pro | 68       |
| 659902             | T         | G      | dpiB (sensor histidine kinase) | Tyr3Asp | 52       |
| 988620             | G         | C      | SEN_RS04610 (hypothetical protein) | Ala89Pro | 130      |
| 104495             | G         | T      | hsd (DNA helicase IV)/Mobile element | Ala204Ser | 75       |
| 1156702            | G         | C      | sirA (virulence gene transcriptional regulator) | Val181Leu | 112      |
| 1325689            | A         | G      | SEN_RS06450 (hydrogenase-1 operon protein HyaF) | Tyr209His | 93       |
| 1427037            | T         | A      | SEN_RS06930 (diguanylate phosphodiesterase) | Asp16Glu | 92       |
| 1787664            | A         | G      | SEN_RS08735 (transporter) | Arg349Gly | 79       |
| 1807289            | G         | A      | SEN_RS08820 (lipoprotein) | Ala14Val | 79       |
| 1931818            | C         | T      | SEN_RS09505 (NAD-dependent deacetylase) | Met37Ile | 82       |
| 2115337            | C         | T      | SEN_RS10585 (cobaalamin biosynthesis protein CbiB) | Gly167Ser | 104      |
| 2419980            | G         | A      | SEN_RS11950 (NADH:ubiquinone oxidoreductase subunit M) | Leu474Phe | 130      |
| 2426844            | A         | G      | SEN_RS11980 (NADH dehydrogenase subunit G) | Val510Ala | 125      |
| 2463887            | T         | C      | SEN_RS12170 (aminoc acid transporter) | Ile512Val | 34       |
| 2647060            | G         | A      | SEN_RS12985 (outer membrane protein RatA) | Pro459Ser | 108      |
| 2647626            | G         | T      | SEN_RS12985 (outer membrane protein RatA) | Ala270Glu | 111      |
| 2672592            | A         | C      | SEN_RS13070 (hypothetical protein) | Ile313Ser | 61       |
| 2956057            | C         | A      | SEN_RS14420 (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase) | Arg539Leu | 123      |
| 3185834            | C         | A      | SEN_RS15495 (D-mannosate oxidoreductase) | Asn515Lys | 81       |
| 3659740            | G         | T      | SEN_RS17815 (membrane protein) | Gln71Lys | 122      |
| 3802073            | G         | A      | coaD (phosphopantetheine adenylyltransferase) | Val118Ile | 127      |
| 4051393            | T         | C      | SEN_RS19620 (DNase TatD) | Ser141Pro | 150      |
| 4059155            | G         | A      | fadB (3-ketoacyl-CoA thiolase) | Ala395Val | 84       |
| 4348398            | A         | G      | SEN_RS20980 (membrane protein)/Salmonella Pathogenicity Island 4 | Asn2902Asp | 158      |
| 4402123            | C         | T      | SEN_RS21190 (sugar:sodium symporter) | Ala395Val | 77       |
| 4476625            | T         | C      | SEN_RS21580 (hypothetical protein) | Lys76Glu | 170      |
| 4555382            | C         | T      | SEN_RS21985 (DNA polymerase III subunit ch) | Asp10Asn | 110      |
used for the interpretation of susceptibility testing results. Quality control was performed using the Escherichia coli ATCC 25922 strain. LV60 isolate was then serotyped by the slide agglutination method for its O and H antigens using the method of Kaufman-White scheme (Grimont and Weill, 2007).

**Whole Genome Sequencing (WGS), Assembly, and Annotation**

Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen), and DNA quantification was performed by Qubit Fluorometric Quantitation (Life Technologies), according to with the manufacturer's instructions. The genome was sequenced using a double strategy of 454 (Roche) and MiSeq (Illumina) sequencing.

Five hundred nanograms of bacterial DNA were fragmented by nebulization, followed by adapter ligation to create double stranded DNA libraries and sequenced on a 454 GS FLX Titanium according to the standard manufacturer's instructions (Roche-454 Life Sciences). The second genome library was prepared from 1 ng of genomic DNA using the Nextera XT DNA Sample Preparation Kit (Illumina) and sequenced on the Illumina MiSeq sequencer (Illumina) using paired-end 2 × 150 bp reads.

First quality evaluation of raw read sequences and their corresponding quality values were assigned by the FastQC software. Reads were then trimmed and filtered according to quality criteria, and de novo assembled with Ray, version 2.3.1 (Boisvert et al., 2010). Contigs were searched for identity by blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nr/nt NCBI database to identify the closest bacterial genome and/or plasmid. Therefore, LV60 genome was mapped against the bacterial genome of S. Enteritidis strain p125109 and its plasmid (NC_011294 and HG970000, respectively) using GS Mapper version 2.9 (Roche). Additionally SNV (single nucleotide variants) and structural variants were also detected with the GS Mapper (Roche, version 2.9).

Structural and functional annotation was performed using PGP (Prokaryotic Genome Prediction) (Egas et al., 2014), an in-house developed pipeline. Taxonomy identification was performed by BLASTP search against the NCBI GenBank non-redundant (nr) database of the 16s rRNA sequence gene, identified in the previous step and confirmed using RNAmer v1.2 (Lagesen et al., 2007).

The final data was submitted in the DDBJ/EMBL/GenBank databases, using the Sequin software tool (http://www.ncbi.nlm.nih.gov/Sequin/). This dataset, which includes files in Genbank (LIHI01.1.gbff.gz), Fasta (LIHI01.1.fasta.gz), and ASN.1 (LIHI01.1.bbs.gz) formats, can be accessed and/or reused at http://www.ncbi.nlm.nih.gov/nuccore/LIHI00000000.

**In silico Analyses**

CLC genomics workbench 8.0 (QIAGEN, Aarhus), PathogenFinder 1.1, ResFinder 2.1, PlasmidFinder 1.3, and MLST 1.8 (MultiLocus Sequence Typing) were used to estimate the number of pathogenicity determinants, acquired antibiotic resistance genes, plasmids and the MLST using the S. Enteritidis genome (Larsen et al., 2012; Zankari et al., 2012; Cosentino et al., 2013; Carattoli et al., 2014). SeqSero tool was used for Salmonella serotyping by whole genome sequencing (Zhang et al., 2015).

PHAST search web tool was applied to detect, identify and annotate prophage sequences (Zhou et al., 2011). ISsaga was used for the high throughput identification and semiautomated annotation of insertion sequences in the genome (Varani et al., 2011). The presence of molecular determinants of antimicrobial resistance was predicted based on homology and SNP models using the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca/analyze/rgi), through Resistance Gene Identifier software (RGI; McArthur et al., 2013).

**RESULTS**

LV60 isolate was serotyped as S. Enteritidis, using the method of Kaufman-White scheme, and found to be wild-type to all the antibiotics tested, except tetracycline.

The de novo assembly yielded 4.977 Mbp distributed in 83 contigs (largest contig with 970,921 bp) with a N50 of 491,005 bp. Overall, the structural and functional annotation with PGP detected 97 tRNA genes, 7 rRNA genes and identified 4656 mRNA genes.

From mapping against the bacterial genome of S. Enteritidis strain p125109, the main difference between the two genomes was the absence of the O-antigen polymerase gene wzy in the LV60 isolate, which in S. Enteritidis is located outside the O antigen gene cluster (Liu et al., 2014). The coding sequence of wzy gene was searched against the assembled genome using blastn, confirming its absence. The flanking regions of wzy gene, which coded for a disrupted membrane and a hypothetical protein, were also absent. The wzy gene is involved in the Wzx/Wzy-dependent pathway, which constitutes the predominant pathway for O-antigen production in Gram-negative bacteria, specifically in *Salmonella* (Hong et al., 2015).

However, in this study, the absence of the wzy gene did not compromise the use of a high-throughput genome sequencing serotype determination method (Zhang et al., 2015), which corroborated the result obtained by the gold standard method. Indeed, this method, based on the detection of O and H antigens encoding genes, predicted an antigenic profile 9:g,m:- based on the O-9,46 wbaV gene, which encodes to the O-antigen tyvelosyl transferase. Furthermore, the S. Enteritidis serotype was confirmed by the presence of sdf gene (*Salmonella* difference fragment virulence gene), a characteristic marker of commonly circulating *S. enterica* serovar Enteritidis (Agron et al., 2001).

Sixty-one SNVs were detected between LV60 and the S. Enteritidis strain p125109. The SNVs that resulted in amino acid substitutions are represented in Table 1. In silico analysis with ResFinder tool did not reveal the presence of any acquired antibiotic resistance genes (90% identity and 40% minimum length) or plasmids (95% identity). However, the RGI analysis, using the perfect algorithm, showed the presence of a Salmonella-specific MerR-like gold (Au) sensor- GolS—influenced in Au resistance (Pontel et al., 2007). This constitutes a matter of concern since antibacterial biocides and metals can contribute...
### TABLE 2 | Perfect and strict best hit results, by predicted gene, obtained using the Resistance Gene Identifier (RGI).

| Predicted gene | e-value | Identity (%) | Contig Average coverage | Start | Stop | RGI Cut-off | RGI Protein Model_type | Antibiotic Resistance Ontology (ARO) category |
|----------------|---------|--------------|-------------------------|-------|------|-------------|------------------------|-----------------------------------------------|
| golS           | 1.41E–108 | 100         | 4                       | 147.97 | 80575 | 81039       | Perfect homolog         | efflux pump conferring AR; chloramphenicol RG; beta-lactam RG; gene modulating antibiotic efflux |
| acrF           | 0       | 99          | 4                       | 147.97 | 73608 | 76775       | Strict homolog          | efflux pump conferring AR; beta-lactam RG; fluoroquinolone RG |
| sdiA           | 0       | 99          | 2                       | 127.7  | 1179091 | 1179813     | Strict homolog          | chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG |
| crp            | 1.30E–151 | 99          | 7                       | 160.37 | 388833 | 389465      | Strict homolog          | efflux pump conferring AR; macrolide RG; beta-lactam RG; gene modulating antibiotic efflux; fluoroquinolone RG |
| mdsA           | 0       | 98          | 4                       | 147.97 | 76772 | 77977       | Strict homolog          | efflux pump conferring AR; chloramphenicol RG; beta-lactam RG |
| mdsC           | 0       | 98          | 4                       | 147.97 | 72134 | 73624       | Strict homolog          | efflux pump conferring AR; chloramphenicol RG; beta-lactam RG |
| aac(6')-Iy     | 2.36E–101 | 97          | 2                       | 127.7  | 808040 | 808477      | Strict homolog          | antibiotic inactivation enzyme; aminoglycoside RG |
| cpxR           | 1.24E–160 | 97          | 3                       | 152.34 | 67603 | 68301       | Strict homolog          | efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux |
| bacA           | 0       | 97          | 14                      | 155.64 | 142061 | 142882      | Strict homolog          | peptide AR gene; gene conferring AR via molecular bypass |
| cpxA           | 0       | 96          | 3                       | 152.34 | 66233 | 67606       | Strict homolog          | efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux |
| baeR           | 5.11E–165 | 96          | 2                       | 127.7  | 107261 | 107983      | Strict homolog          | efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux |
| emrY           | 0       | 95          | 8                       | 158.13 | 93935 | 95473       | Strict homolog          | efflux pump conferring AR; tetracycline RG |
| marA           | 1.35E–82 | 95          | 2                       | 127.7  | 702301 | 702690      | Strict homolog          | chloramphenicol RG; gene modulating antibiotic efflux; gene modulating permeability to antibiotic; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG |
| H-NS           | 9.89E–75 | 94          | 2                       | 127.7  | 965098 | 965511      | Strict homolog          | gene modulating antibiotic efflux; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; beta-lactam RG |
| mexD           | 0       | 94          | 5                       | 135.43 | 37513 | 40626       | Strict homolog          | chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG |
| phoP           | 6.18E–151 | 93          | 2                       | 127.7  | 417112 | 417786      | Strict homolog          | efflux pump conferring AR; polymyxin RG; macrolide RG; gene modulating antibiotic efflux; gene altering cell wall charge conferring AR |
| envrR          | 7.58E–115 | 93          | 8                       | 158.13 | 92089 | 92619       | Strict homolog          | efflux pump conferring AR; gene modulating antibiotic efflux; fluoroquinolone RG |
| mexD           | 0       | 93          | 4                       | 147.97 | 209028 | 212177      | Strict homolog          | chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG |
| mdtH           | 0       | 92          | 2                       | 127.7  | 349496 | 350704      | Strict homolog          | efflux pump conferring AR |
| mdtK           | 0       | 92          | 2                       | 127.7  | 607306 | 608679      | Strict homolog          | efflux pump conferring AR; fluoroquinolone RG |
| mexN           | 0       | 92          | 2                       | 127.7  | 113873 | 116995      | Strict homolog          | efflux pump conferring AR; chloramphenicol RG |

(Continued)
### TABLE 2 | Continued

| Predicted gene | e-value | Identity (%) | Contig | Average coverage | Start | Stop | RGI Cut-off | RGI Protein Model_type | Antibiotic Resistance Ontology (ARO) category |
|----------------|---------|--------------|-------|------------------|-------|------|-------------|-----------------------|-----------------------------------------------|
| mexN           | 0       | 91           | 2     | 127.7            | 110792| 113872| Strict      | homolog               | efflux pump conferring AR; chloramphenicol RG |
| emrD           | 0       | 90           | 7     | 160.37           | 11534 | 12718 | Strict      | homolog               | efflux pump conferring AR                     |
| mdtG           | 0       | 90           | 2     | 127.7            | 339682| 340896| Strict      | homolog               | polyoxyn RG; gene altering cell wall charge conferring AR |
| pmrA           | 1.77E–143 | 90          | 9     | 160.96           | 119082| 119750| Strict      | homolog               | chloramphenicol RG; gene modulating antibiotic efflux |
| emrA           | 0       | 89           | 8     | 158.13           | 92719 | 93918 | Strict      | homolog               | efflux pump conferring AR; fluoroquinolone RG |
| pmrE           | 0       | 89           | 2     | 127.7            | 174573| 175739| Strict      | homolog               | polyoxyn RG; gene altering cell wall charge conferring AR |
| baeS           | 0       | 89           | 2     | 127.7            | 107980| 109383| Strict      | homolog               | efflux pump conferring AR; aminocoumarin RG; aminoglycoside RG; gene modulating antibiotic efflux |
| talC           | 0       | 89           | 14    | 155.64           | 163404| 164879| Strict      | homolog               | chloramphenicol RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; aminocoumarin RG; tetracycline RG; rifampin RG; beta-lactam RG |
| acrE           | 0       | 88           | 1     | 155.02           | 4223  | 5380 | Strict      | homolog               | efflux pump conferring AR; beta-lactam RG; fluoroquinolone RG |
| mexD           | 0       | 88           | 1     | 155.02           | 1098  | 4211 | Strict      | homolog               | chloramphenicol RG; trimethoprim RG; macrolide RG; fluoroquinolone RG; efflux pump conferring AR; beta-lactam RG |
| mdtA           | 0       | 87           | 13    | 131.07           | 105101| 106333| Strict      | homolog               | efflux pump conferring AR                     |
| pmrF           | 0       | 87           | 5     | 135.43           | 231615| 232598| Strict      | homolog               | polyoxyn RG; gene altering cell wall charge conferring AR |
| mdtM           | 0       | 86           | 11    | 163.1            | 148308| 149549| Strict      | homolog               | efflux pump conferring AR                     |
| ramA           | 1.93E–71 | 86          | 4     | 147.97           | 311233| 311622| Strict      | homolog               | chloramphenicol RG; gene modulating antibiotic efflux; gene modulating permeability to antibiotic; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG |
| mdtD           | 0       | 86           | 2     | 127.7            | 109383| 110795| Strict      | homolog               | efflux pump conferring AR                     |
| acrA           | 0       | 85           | 4     | 147.97           | 212200| 213393| Strict      | homolog               | chloramphenicol RG; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG |
| phoQ           | 0       | 85           | 2     | 127.7            | 415649| 417112| Strict      | homolog               | efflux pump conferring AR; polyoxyn RG; macrolide RG; gene modulating antibiotic efflux; gene altering cell wall charge conferring AR |
| pmrB           | 0       | 85           | 9     | 160.96           | 118002| 119081| Strict      | homolog               | polyoxyn RG; gene altering cell wall charge conferring AR |
| mdtA           | 0       | 82           | 2     | 127.7            | 116995| 118332| Strict      | homolog               | efflux pump conferring AR; aminocoumarin RG |
| pmrC           | 0       | 82           | 9     | 160.96           | 119747| 121390| Strict      | homolog               | polyoxyn RG; gene altering cell wall charge conferring AR |
| acrR           | 1.83E–124 | 82          | 4     | 147.97           | 213535| 214188| Strict      | variant               | chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; antibiotic resistant gene variant or mutant; tetracycline RG; rifampin RG; beta-lactam RG |
| robA           | 0       | 81           | 11    | 163.1            | 77518 | 78387 | Strict      | homolog               | chloramphenicol RG; gene modulating antibiotic efflux; fluoroquinolone RG; efflux pump conferring AR; tetracycline RG; rifampin RG; beta-lactam RG |
| amnA           | 0       | 79           | 5     | 135.43           | 229636| 231618| Strict      | homolog               | polyoxyn RG; gene altering cell wall charge conferring AR |

(Continued)
TABLE 2 | Continued

| Predicted gene | e-value | Identity (%) | Contig Average coverage | Start | Stop | RGI Cut-off | RGI Protein Model_type | Antibiotic Resistance Ontology (ARO) category |
|----------------|---------|--------------|-------------------------|-------|------|------------|------------------------|-----------------------------------------------|
| mdtL           | 0       | 77           | 16                      | 156.65| 44691| 45878      | Strict homolog          | efflux pump conferring AR                      |
| rosB           | 0       | 74           | 4                       | 147.97| 230248| 231924     | Strict homolog          | polymyxin RG                                  |
| rosA           | 0       | 71           | 4                       | 147.97| 232128| 233348     | Strict homolog          | efflux pump conferring AR; polymyxin RG       |
| rpoB           | 0       | 58           | 19                      | 154.2 | 4220  | 8248       | Strict variante         | rifampin; antibiotic resistant gene variant or mutant |
| katG           | 0       | 56           | 3                       | 152.34| 121560| 123740     | Strict variant          | antibiotic resistant gene variant or mutant; isoniazid RG |
| gyrB           | 0       | 55           | 16                      | 156.65| 54369 | 56783      | Strict homolog          | aminocoumarin RG; antibiotic resistant gene variant or mutant |
| macB           | 0       | 50           | 13                      | 131.07| 143618| 145564     | Strict homolog          | efflux pump conferring AR; macrolide RG       |
| vanG           | 8.15E−81| 38           | 4                       | 147.97| 113335| 114447     | Strict homolog          | glycopeptide RG; AR gene cluster, cassette, or operon; gene conferring AR via molecular bypass |
| macA           | 2.30E−51| 35           | 13                      | 131.07| 142503| 143621     | Strict homolog          | efflux pump conferring AR; macrolide RG       |

RGI, resistance gene; AR, antibiotic resistance.

to the development and maintenance of antibiotic resistance in bacterial communities through mechanisms of cross- or co-resistance (Baker-Austin et al., 2006; Lemire et al., 2013; Pal et al., 2015).

Furthermore, the RGI strict algorithm, which detects previously unknown variants of known antimicrobial resistance genes, identified 52 genes involved in efflux, transport, and permeability, which might justify the low-level tetracycline resistance identified by phenotypic methods (Table 2). Resistance to additional classes of antibiotics such as fluoroquinolones, aminoglycosides, and chloramphenicol were bioinformatically predicted. Indeed, efflux pumps are often associated with discrete decreases in antibiotic susceptibility that may not necessarily reflect an alteration in interpretation categories (Fernández and Hancock, 2012). Genes responsible for the intrinsic resistance to benzylpenicillin, glycopeptides, macrolides, and rifampicin were also detected.

The total number of pathogenicity determinants present in the genome of S. Enteritidis LV60, matching 1164 pathogenic families, showed a 94.1% certainty of the isolate being a human pathogen. Here we highlight the presence of Salmonella Pathogenicity Island 4, which usually encodes a non-fimbrial adhesion and the cognate type 1 secretion system (Gerlach et al., 2007).

The use of complementary web tools assigned this isolate to ST11, which according with MLST data (http://mlst.warwick.ac.uk/) is commonly found among CTX-M-14 and CTX-M-15-producing S. Enteritidis human isolates (Kim et al., 2011; Bado et al., 2012). In this study, the identification of ST11 in an isolate of animal origin, together with other pathogenicity determinants may suggest its zoonotic potential.

We also identified 6 prophage regions, among which three were incomplete and three were intact. The last included prophage regions reaching the lengths of 64.3, 49.2, and 31.7 Kb, and encoding 42, 78, and 66 DNA coding sequences, respectively.

Overall, 33 different IS were detected within the genome, which were distributed as follows: 27.03% of IS3 family, 18.92% of IS256 family, 13.51% of IS unclassified family, 10.81% of IS200/IS605 complex, and of ISL3 family, 8.11% of IS481 family, 5.41% of IS630 family, and 2.7% of IS1 and IS110 families. All identified structures (pathogenicity island, prophages, ISs) constitute a multiplicity of pathogenicity factors in LV60 S. Enteritidis isolate and contribute for the fitness of the isolate in different environments; its presence may also suggest the possibility of acquisition of other factors by different mechanisms, including resistance genes e.g., by horizontal gene transfer, contributing to its biological diversity and genetic evolution.

CONCLUSION

The detection of an avian S. Enteritidis isolate harboring multiple efflux pumps, pathogenicity factors, a variety of mobile genetic elements and heavy-metal-tolerance genes raises concerns regarding the dissemination of infection in birds and potential risk of zoonotic transmission.

This study demonstrated the added value of WGS as a routine tool for surveillance programs directed to food-producing animals, which might complement sanitary measures, essential to prevent the spread of Salmonella infections among animals. It also proved to have an added value as a complementary typing method. Moreover, the simultaneous detection of putative Au resistance, intrinsic antibiotic resistant genes, and mobile genetic elements, underline this method as a helpful resource to follow the spread and evolution of antibiotic resistance in this species by genomic comparison studies.

DATA ACCESS

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession LIHI00000000. The version described in this paper is version LIHI01000000.
AUTHOR CONTRIBUTIONS

DJ designed the study, performed molecular experiments, analyzed the data and wrote the manuscript. LC performed the microbiological experiments and reviewed the manuscript. CE, HF performed 454 Roche genome sequencing experiments and analyze the data; DS, LV performed Illumina genome sequencing experiments. MF, NT analyzed the data. VM designed the study, analyzed the data and reviewed the manuscript. MC designed the study, reviewed and edited the manuscript. All authors read and approved the final manuscript.

REFERENCES

Agron, P. G., Walker, R. L., Kinde, H., Sawyer, S. J., Hayes, D. C., Wollard, J., et al. (2001). Identification by subtractive hybridization of sequences specific for Salmonella enterica serovar Enteritidis. Appl. Environ. Microbiol. 67, 4984–4991. doi: 10.1128/AEM.67.11.4984-4991.2001
Bado, I., García-Fulgueiras, V., Cordeiro, N. F., Betancor, L., Caiata, L., Seija, V., et al. (2012). First human isolate of Salmonella enterica serotype Enteritidis harboring blaCTX-M-14 in South America. Antimicrob. Agents Chemother. 56, 2132–2134. doi: 10.1128/AAC.05530-11
Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-Bado, I., García-Fulgueiras, V., Cordeiro, N. F., Betancor, L., Caiata, L., Seija, V., et al. (2012). First human isolate of Salmonella enterica serotype Enteritidis harboring blaCTX-M-14 in South America. Antimicrob. Agents Chemother. 56, 2132–2134. doi: 10.1128/AAC.05530-11
Baker-Austin, C., Wright, M. S., Stepanauskas, R., and McArthur, J. V. (2006). Co-Bado, I., García-Fulgueiras, V., Cordeiro, N. F., Betancor, L., Caiata, L., Seija, V., et al. (2012). First human isolate of Salmonella enterica serotype Enteritidis harboring blaCTX-M-14 in South America. Antimicrob. Agents Chemother. 56, 2132–2134. doi: 10.1128/AAC.05530-11

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Foley, S. L., and Lynne, A. M. (2008). Food animal-associated Salmonella challenges: pathogenicity and antimicrobial resistance. J. Anim. Sci. 86, E173–E187. doi: 10.2527/jas.2007-0447
Foley, S. L., Lynne, A. M., and Nayak, R. (2008). Salmonella challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. J. Anim. Sci. 86, E149–E162. doi: 10.2527/jas.2007-0464
Gerlach, R. G., Jackel, D., Stecher, B., Wagner, C., Lupsas, A., Hardt, W. D., et al. (2007). Salmonella Pathogenicity Island 4 encodes a giant non-fimbrial adhesin and the cognate type 1 secretion system. Cell Microbiol. 9, 1834–1850. doi: 10.1111/j.1462-5822.2007.00919.x
Grimont, P. A., and Weill, F. X. (2007). Antigenic Formulæ of the Salmonella Serovars, 9th Edn. Paris: Institute Pasteur; WHO Collaborating Centre for Reference and Research on Salmonella.
Hensel, M. (2004). Evolution of pathogenicity islands of Salmonella enterica. Int. J. Med. Microbiol. 294, 95–102. doi: 10.1016/j.ijmm.2004.06.025
Hong, Y., Morcilla, V., A., Liu, M. A., Russell, E. L., and Reeves, P. R. (2015). Three Way polymers are specific for particular forms of an internal linkage in otherwise identical O units. Microbiology 161, 1639–1647. doi: 10.1099/mic.0.000113
Kim, Y., Bae, I. K., Jeong, S. H., Lee, C. H., Lee, H. K., Ahn, J., et al. (2011). Occurrence of extended-spectrum β-lactamases among isolates of Salmonella enterica subsp. enterica from food-producing animals and food products, in Portugal. Int. J. Food Microbiol. 167, 221–228. doi: 10.1016/j.ifm.2013.08.009
Clemente, L., Clemente, L., Manageiro, V., Ferreira, E., Jones-Dias, D., Correia, I., Themudo, P., et al. (2013). Occurrence of extended-spectrum β-lactamases among isolates of Salmonella enterica subsp. enterica from food-producing animals and food products, in Portugal. Int. J. Food Microbiol. 167, 221–228. doi: 10.1016/j.ifm.2013.08.009
Clemente, L., Manageiro, V., Jones-Dias, D., Correia, I., Themudo, P., Albuquerque, T., et al. (2015). Antimicrobial susceptibility and oximino-β-lactam resistance mechanisms in Salmonella enterica and Escherichia coli isolates from different animal sources. Res. Microbiol. 166, 574–583. doi: 10.1016/j.resmic.2015.05.007
Cosentino, S., Voldby Larsen, M., Møller Aarestrup, F., and Lund, O. (2013). PathogenFinder-distinguishing friend from foe using bacterial whole genome sequence data. PLoS ONE 8:e77302. doi: 10.1371/journal.pone.0077302
Delmar, J. A., Su, C. C., and Yu, E. W. (2014). Bacterial multidrug efflux transporters. Annu. Rev. Biophys. 43, 93–117. doi: 10.1146/annurev-biophys-051013-022855
Egas, C., Barroso, C., Froufe, H. J., Pacheco, J., Albuquerque, L., and Da Costa, M. S. (2014). Complete genome sequence of the radiation-resistant bacterium Rubrobacter radiotolerans RSPS-4. Stand. Genomic Sci. 9, 1062–1075. doi: 10.4056/sigs.5661021
European Food Safety Authority/European Center Disease Control (EFSA/ECDC) (2015). EU Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. EFSA J. 13, 4036. doi: 10.2903/j.efsa.2015.4036
Fernández, L., and Hancock, R. E. W. (2012). Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin. Microbiol. Rev. 25, 661–681. doi: 10.1128/CMR.00043-12
Foley, S. L., Johnson, T. J., Rieke, S. C., Nayak, R., and Danzeisen, J. (2013). Salmonella pathogenicity and host adaptation in chicken-associated serovars. Microbiol. Mol. Biol. Rev. 77, 582–607. doi: 10.1128/MMBR.00015-13
novel insights into their co-selection potential. BMC Genomics 16:964. doi: 10.1186/s12864-015-2153-5

Pontel, L. B., Audero, M. E. P., Espariz, M., Checa, S. K., and Soncini, F. C. (2007). GolS controls the response to gold by the hierarchical induction of Salmonella-specific genes that include a CBA efflux-coding operon. Mol. Microbiol. 66, 814–825. doi: 10.1111/j.1365-2958.2007.05963.x

Poole, K. (2004). Efflux-mediated multiresistance in Gram-negative bacteria. Clin. Microbiol. Infect. 10, 12–26. doi: 10.1111/j.1469-0691.2004.00763.x

Varani, A. M., Siguier, P., Gourbeyre, E., Charneau, V., and Chandler, M. (2011). ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. Genome Biol. 12, R30. doi: 10.1186/gb-2011-12-3-r30

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 67, 2640–2644. doi: 10.1093/jac/dks261

Zhang, S., Yin, Y., Jones, M. B., Zhang, Z., Deatherage Kaiser, B. L., Dinsmore, B. A., et al. (2015). Salmonella serotype determination utilizing high-throughput genome sequencing data. J. Clin. Microbiol. 53, 1685–1692. doi: 10.1128/JCM.00323-15

Zhou, Y., Liang, Y., Lynch, K. H., Dennis, J. J., and Wishart, D. S. (2011). PHAST: a fast phage search tool. Nucleic Acids Res. 39, W347–W352. doi: 10.1093/nar/gkr485

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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