Diverse distribution of Toxin-Antitoxin II systems in Salmonella enterica serovars

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Type II Toxin-Antitoxin systems (TAs), known for their presence in virulent and antibiotic resistant bacterial strains, were recently identified in Salmonella enterica isolates. However, the relationships between the presence of TAs (ccdAB and vapBC) and the epidemiological and genetic features of different non-typhoidal Salmonella serovars are largely unknown, reducing our understanding of the ecological success of different serovars. Salmonella enterica isolates from different sources, belonging to different serovars and epidemiologically unrelated according to ERIC profiles, were investigated for the presence of type II TAs, plasmid content, and antibiotic resistance. The results showed the ubiquitous presence of the vapBC gene in all the investigated Salmonella isolates, but a diverse distribution of ccdAB, which was detected in the most widespread Salmonella serovars, only. Analysis of the plasmid toxin ccdB translated sequence of four selected Salmonella isolates showed the presence of the amino acid substitution R99W, known to impede in vitro the lethal effect of CcdB toxin in the absence of its cognate antitoxin CcdA. These findings suggest a direct role of the TAs in promoting adaptability and persistence of the most prevalent Salmonella serovars, thus implying a wider eco-physiological role for these type II TAs.

Salmonella spp. are the second most frequent zoonotic agent in the European Union (EU)1 and represent a major challenge for animal health and food safety because of their high endemicity and morbidity rate, and of the difficulty in controlling the pathogen2. Salmonellosis is also the most common foodborne illness in the United States (US)3, causing the largest number of deaths and having the highest cost burden. Majowicz et al.4 estimated that 93.8 million cases of gastroenteritis due to Salmonella species occur globally each year, of which 80.3 million are foodborne, and which cause 155,000 deaths. In the EU, in 2013, 22.5% of all food-borne outbreaks were related to Salmonella, and 82,694 confirmed cases of human salmonellosis were reported1. Salmonella enterica serovar Enteritidis and Salmonella enterica serovar Typhimurium (plus its monophasic variant S. 1,4,[5],12:i:-) are considered of paramount public health significance in the EU, as they accounted for 39.5%, 20.2%, and 8.6%, respectively, among all reported serovars in confirmed human infections in 20131. The occurrence of S. 1,4,[5],12:i:- is constantly increasing over time throughout the EU1,5,6. US data from food-borne outbreaks related to human illness, collected over the period 2007–2011, confirmed that S. Enteritidis was the predominant serovar followed by S. Typhimurium7. In Italy, the picture is slightly different, since in the last few years S. 4,[5],12:i:- has become the predominant serovar responsible for human infection followed by S. Typhimurium, whereas S. Enteritidis has been displaying a marginal role in terms of public health8. In animals, non-motile S. Gallinarum is a host specific serovar, which causes great concern due to its high infectivity9.

There is much evidence suggesting that only a few Salmonella serovars are responsible for the vast majority of human infection cases, but, at the same time, it is unclear why these common serovars might have greater ecological success. A combination of different factors specific to each serovar, such as the presence of virulence plasmids, the cell surface structure, the presence of flagellin genes and the Salmonella pathogenicity islands, can be associated with the severity of human Salmonella infections10. Studies conducted to date have revealed that host range

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and virulence of *Salmonella* serovars are evolutionarily related to gene acquisition by horizontal transfer of mobile elements and by the loss of genes or gene functionality11.

Following these considerations, great interest exists in reducing the *Salmonella* impact on human and animal health by lowering its prevalence along the food chain through the implementation of control measures from farm to fork.

A deeper knowledge of the eco-evolutionary mechanisms enabling some *Salmonella* serovars to be widespread will lead to a better understanding of the epidemiology of virulence and finally to the design of more efficient control actions.

Widespread and virulent *Salmonella* serovars, such as *S. Typhimurium* and *S. 4,[5],12:i:-*, frequently exhibit a higher frequency of multidrug resistance phenotype than rare and less virulent serovars5,12,13. Furthermore, the genes for antibiotic resistance often occur on incompatible plasmids14. Many of these plasmids encode addiction systems, and harbour toxin-antitoxin (TA) factors, thus promoting post-segregation killing to maintain themselves15. The permanence of these specific plasmids in the bacterial population is promoted by the action of TA systems, regardless of other selective pressures15,16.

In particular, the type II TA modules, a pair of genes encoding a protein that interferes with basic cell metabolism (the toxin) and its antagonist (the antitoxin), are generally considered as genetic elements involved in bacterial stress response activities via a persistence mechanism17. Furthermore, the involvement of TA systems in a wide range of biological functions including growth control, defense against phages, and biofilm formation has been highlighted by recent research18-20.

Analysis of the *Salmonella* genome revealed the presence of 24 *Salmonella* TA loci21,22 accounting for 5 type I and 19 type II TA modules, most of which showed a narrow distribution restricted to pathogenic *Salmonella enterica*, and which were missing in poor pathogenic species *S. bongori*23. This observation led Lobato-Márquez and co-workers to hypothesize an active role for TA modules in enhancing the fitness of pathogenic *Salmonella* inside eukaryotic cells21.

The current study is focused on two type II TA systems, *vapBC* and *ccdAB*, associated with virulence in *Salmonella* and for which no clear evidence on their diffusion among virulent and widespread *Salmonella* serovars exists to date.

*Vap*BC (virulence associated protein) locus, originally called *vagCD*23 was identified for the first time on a *S. Dublin* virulence plasmid24. *VapC* was classified as a PIN domain ribonuclease, and originally reported to inhibit translation by cleaving the initiator tRNAfMet25.

*CcdAB* (coupled cell division locus) has been described as involved in plasmid maintenance26. In the case of *S. Typhimurium*, it was mapped on the virulence plasmid pSLT21. The *ccdB* gene encodes a 101 amino acid residue protein able both to bind free DNA gyrase and the DNA-gyrase complexes, thus inhibiting DNA gyrase activity and/or trapping the gyrase-DNA cleavable complex; this causes DNA lesions, leading to the formation of lethal double strand DNA breaks. Moreover, the DNA gyrase inhibition, in the context of the gyrase-DNA complex, is suppressed by mutations occurring in the last three amino acid residues of CcdB that, in turn, inactivate the killing phenotype of CcdB26-12.

This study aimed to further our understanding of the eco-physiological role for these type II TAs, on a variety of isolates belonging to different serovars, including serovars which are commonly associated with human infections (*S. Typhimurium*, *S. Enteritidis*, *S. 4,[5],12:i:-*), species-specific serovars such as *S. Gallinarum*, and some other serovars, which have been rarely or never related to documented cases of infection, neither in animals nor in humans (*S. Tennessee*, *S. Lawndale* and *S. Alachua*). The presence of type II TAs in the different *Salmonella* serovars was thus examined in relation to the isolates’ sources, specific antibiotic resistance, and plasmid profile.

**Results**

**Isolates selection.** The 45 isolates (Fig. 1) comprised *S. 4,[5],12:i:-* (n = 18), *S. Typhimurium* (n = 15), *S. Enteritidis* (n = 5), *S. Gallinarum* (n = 3) and one each of *S. Tennessee*, *S. Lawndale* and *S. Alachua*. The last three isolates, serovars: *Tennessee*, *Lawndale* and *Alachua*, have been only rarely isolated from humans; thus, they were included in the study as a comparison with the other widespread and prevalent serovars.

*S. Lawndale* and *S. Alachua*, to our knowledge, have never been involved in outbreaks. *S. Tennessee* was responsible for a few cases of infections associated with powdered milk products and infant formula33 or peanut butter34 in the USA and Canada, respectively.

The sources of the *Salmonella* isolates were: 15 from swine, 11 from chicken, 4 from bovine, 2 from turkey, 9 from humans and the remaining 4 from other origins (vegetables, reptile, cat and rabbit).

*S. Typhimurium* and *S. 4,[5],12:i:-* isolates were selected as they belonged to the five most common phage-types isolated in Italy (DT193, U311, DT120, U302 and DT104). *S. Enteritidis* isolates displayed the phage-types: PT4, PT6, PT8 and RDNC (Fig. 1).

**ERIC-PCR typing profiles**. The 45 examined isolates showed genetic diversity. The isolates were grouped into two major clusters having less than 70% sample match similarity. The first cluster included all the *S. Typhimurium* and *S. 4,[5],12:i:-* isolates, which are known to be two closely related serovars8, whereas the second cluster included isolates of *S. Enteritidis*, *S. Gallinarum* and the rare serovars, *S. Tennessee*, *S. Lawndale* and *S. Alachua* (Fig. 1). Different sub-groups were identified within each of the two principal clusters. *S. Typhimurium* and *S. 4,[5],12:i:-* were grouped into five sub-clusters, while serovars belonging to the second cluster were assorted into two sub-clusters, irrespective of their sources of isolation. One isolate each of *S. Gallinarum* and *S. Enteritidis* did not sub-cluster with any other *Salmonella* isolates (Fig. 1). The rare serovars *S. Tennessee* and *Alachua* were sub-clustered together with two *S. Enteritidis* isolates, while *S. Lawndale* was found to be outside of any cluster (Fig. 1). In some cases, isolates belonging to the same serovar were grouped into different sub-clusters, and some sub-clusters included isolates belonging to different serovars.
Antimicrobial Susceptibility Assessment. The phenotypic resistances of the *Salmonella* isolates to antimicrobials are shown in Fig. 1, and reveal various patterns of resistance. Specific resistances to ampicillin (A), trimethoprim (Tr), florfenicol (F), chloramphenicol (Ch) and tetracycline (T) were found exclusively among *S. Typhimurium* and *S. 4,[5],12:i:-*, which were also the serovars most frequently resistant to streptomycin (S), and sulfamethoxazole (Su). Four out of the six *S. Enteritidis* isolates were resistant only to Su (1 isolate) or Colistin (Col) (3 isolates), with the latter resistance also being shown by all the *S. Gallinarum* isolates. Resistance to quinolones and fluoroquinolones, nalidixic acid (Na) and ciprofloxacin (Cip), were displayed only by *S. Typhimurium* (4 out of 15 isolates) and *S. Gallinarum* (2 out of 3). The remaining serovars, *S. Tennessee*, *S. Alachua* and *S. Lawndale* were pansusceptible to all antimicrobials examined.

Moreover, half of the *S. 4,[5],12:i:-* isolates displayed a typical profile of tetra-resistance to A, S, Su and T. Eleven *S. Typhimurium* isolates were also resistant to these four antimicrobials, although they generally displayed resistances to other antimicrobials as well.

In order to get deeper insight into the transferability of the antimicrobial resistance patterns displayed by the selected isolates, molecular screening for the most common resistance genes (ARGs) was performed. The ARGs, *bla*TEM, *strA* and *sulII*, were commonly associated to different extents with serovars *Typhimurium*, *4,[5],12:i:-*, *Enteritidis* and *Gallinarum*. In addition, some *S. Typhimurium* and *S. 4,[5],12:i:-* isolates contained *tetB* and *sulII* genes (Fig. 1). In contrast, DNA belonging to *S. Tennessee* and *S. Lawndale* displayed *tet*(A), only. The *T* resistance gene (*tet*(A)) was also present in one *S. Typhimurium* and three *S. 4,[5],12:i:-* isolates. Finally, none of the examined genes was harbored by the *S. Alachua* isolate. The antimicrobial resistance genes *qnr*A, *qnr*S and *bla*SHV were not detected in any of the examined isolates.

Inc-plasmid molecular typing. PCR-based replicon typing was applied to type the incompatibility (Inc) plasmids harbored by the investigated isolates. A variety of Inc-plasmids were present in *S. 4,[5],12:i:-* and in *S. Typhimurium* isolates, as the former harbored HI2, FIIS, FII and XI and the latter HI2, FIIS and P plasmids (Fig. 1). In particular, HI2 plasmids were harbored by all *S. 4,[5],12:i:-* and *S. Typhimurium* strains belonging...
ily44 as carriers of virulence and antibiotic resistance determinants. Moreover, they display multiple alleles in the pattern was observed. Concerning IncF plasmids, they have been found only in the material. In fact, IncHI2 and IncFIIS replicons are common in their widespread presence suggests that they might have a role in the occurrence of this plasmid was significantly lower in the Typhimurium and 4,12:i-:serovars compared to the other investigated serovars (p < 0.05).

**Screening for the presence of type II TA modules.** Different patterns of presence of the vapBC and ccdAB TA modules characterized the DNA extracted from the Salmonella isolates. vapBC was constitutively found in every examined isolate, while ccdAB was present only in S. Typhimurium, S. Enteritidis and S. Gallinarum serovars, but was absent in the rare serovars Tennessee, Lawndale and Alachua (Fig. 1). S. 4,12:i:- strains harbored ccdAB with the exception of 4 strains (SAL2533_1, 2011_863, 2011_32981 and 2014_77), all belonging to sub-group e (Fig. 1).

**CcdB sequence analysis.** Comparison between the CcdB protein sequence of the isolates S. 4,12:i:-:864_2013, S. Enteritidis 2011_2256/1, S. Enteritidis 2012_1058/2 and S. Typhimurium 149_2014 and of E. coli revealed numerous amino acid substitutions in these Salmonella isolates (Fig. 2) among which the R99W mutation was present in all examined Salmonella. Residue W99 is reported to be crucial for CcdB toxicity in E. coli.

**Discussion**

In this study, the spread of type II TAs, in relation to source, specific antibiotic resistance, and plasmid profile was studied through analysis of 45 Salmonella isolates belonging to highly pathogenic serovars, both for humans and animals, as well as serovars which have been rarely associated with infection episodes. The isolates were tested for their heterogeneity by means of ERIC-PCR subtyping. In fact, by complementing phenotypic data, ERIC-PCR has been demonstrated as allowing rapid and cost effective exploration of the genetic relatedness among any one Salmonella serovar and thus being a rapid tool to broadly investigate genetic relatedness among isolates.

The close relationship between S. Typhimurium and S. 4,12:i:- was confirmed; in consequence, all isolates belonging to these two serovars were included in the same cluster and were clearly differentiated from all other investigated serovars. The other serovars studied were grouped in a second cluster, including two serologically related serovars (i.e. S. Enteritidis and S. Gallinarum), and other serovars that did not share somatic and/or flagellar antigens. Within this cluster, isolates of the same serovar were then included in different sub-groups and each sub-group included isolates belonging to different serovars. The frequency of antimicrobial resistance phenotypes was higher among S. Typhimurium and S. 4,12:i:- isolates in comparison to the other investigated serovars, with tetra-resistance “ASSuT” being the predominant multiple drug resistance profile in these two serovars.

The presence of ASSuT has become important in Italy since the year 2000, being increasingly detected both in S. Typhimurium and S. 4,12:i:-. Moreover, S. Typhimurium isolates are more frequently resistant to F and Ch, while these resistances appear to be less common in S. 4,12:i:-.

A low level of antimicrobial resistance, as previously described by other authors, was found for S. Enteritidis, even though it is known to be one of the most common serovars in human infections. To investigate the plasmid content and thus the potential mobilization of ARGs, Salmonella isolates were examined for the presence of the major Inc plasmid groups by replicon type analysis. The results showed the presence of a heterogeneous panel of plasmids in the epidemiologically unrelated S. Typhimurium and S. 4,12:i:- isolates. The observed variability of plasmid content among the examined isolates highlights the diversity of the Salmonella serovars. IncHI2 and IncFIIS were the most common plasmid groups among the investigated isolates. Their widespread presence suggests that they might have a role in Salmonella acquiring and transferring genetic material. In fact, IncHI2 and IncFIIS replicons are common in Enterobacteriaceae and have been recently associated with highly disseminated β-lactamase genes in E. coli and Salmonella from food animals.

IncHI2 plasmids belonging to the major IncHI group are usually associated with human pathogens and carry antibiotic and heavy metal resistance genes. In the investigated isolates, IncHI2 plasmids were present only in S. Typhimurium and S. 4,12:i:-: belonging to three diverse sub-clusters thus suggesting a direct influence of the phylogeny of the strains in the acquisition/permanence of specific Inc plasmid groups.

No significant relationship between the presence of IncHI2 plasmids and any specific antibiotic resistance pattern was observed. Concerning IncF plasmids, they have been found only in the Enterobacteriaceae family as carriers of virulence and antibiotic resistance determinants. Moreover, they display multiple alleles for each of the replicons FII, FIA, FIB and FIC. FII is a subgroup of the IncFI replicon which is strictly associated with Salmonella spp. The occurrence of IncFIIS plasmids in Salmonella isolates of various serovars in our study highlights their tendency to spread. Moreover, the absence of these replicons in the rare Salmonella serotypes Tennessee, Alachua and Lawndale evokes their involvement in the epidemiology of Salmonella. However, the presence of this plasmid was significantly associated with serovars other than Typhimurium and...
and colleagues (2013) who found virulent serovars of Salmonella only rarely have been linked to human salmonellosis. This finding confirms the observations made by De la Cruz the constitutive occurrence of low plasmid copy-number as described by the supplier. Miniprep Kit (Qiagen) and by applying the modifications to the protocols required for the isolation of high and the classical boiling method, while the plasmid DNA was obtained from pelleted cells using the QIAprep Spin Phage Typing (IFEPT, Laboratory of Enteric Pathogens, Health Protection Agency, Colindale, London, UK). 

A closer look at the distribution of vapBC and ccdAB in the serovars examined in the present study revealed the constitutive occurrence of vapBC in all isolates, coupled with the more limited presence of the ccdAB locus. This locus was mainly restricted to widespread isolates, and was missing in the three isolates of serovars which only rarely have been linked to human salmonellosis. This finding confirms the observations made by De la Cruz and colleagues (2013) who found virulent serovars of S. enterica displaying multiple TA systems, in contrast to less-pathogenic isolates which harbored no or low numbers of TA modules.

S. Enteritidis, S. Typhimurium, and S. 4,5,12:i- are the three most prevalent serovars in Europe; thus, the widespread occurrence of both the TA operons among their genomes, as demonstrated by the present study, might represent a selective advantage supporting their successful diffusion.

The complete sequencing of S. Typhimurium strain LT2 revealed the presence of differences in the CcdB codons compared to its E. coli active ortholog. In particular, one of the differences was the R99W amino acid substitution, known to compromise the lethal effect of CcdB, when expressed in S. Typhimurium in the absence of its cognate antitoxin CcdA. Our results confirmed the presence of the same substitution in the Salmonella isolates examined, as an Arginine was coded in the 99th amino acid position. This suggests the possibility that in Salmonella, plasmid toxin CcdB could play a different role than that of post-segregational killing, and instead, it could be connected to the activation of persistent phenotypes. This could represent an advantage for the most prevalent Salmonella serovars to adapt to different ecological niches and to resist changing environmental conditions.

Methods

Salmonella isolates. The Salmonella isolates studied are reported in Fig. 1. Salmonella isolates from animals and foodstuffs were collected by the OIE Reference Laboratory for Salmonellosis (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy) over the period 2010–2015. Human-derived Salmonella isolates were kindly provided by Dr. Ida Luzzi (Istituto Superiore di Sanità, Rome, Italy).

Salmonella isolates were grouped on the basis of their importance for public health and according to the following criteria: (i) originating from animal, foodstuff or human sources; (ii) representative of different animals species and foodstuff type; (iii) belonging to invasive, virulent and widespread serovars such as Enteritidis, Typhimurium, 4,5,12:i- or Gallinarum; (iv) representative of different phage types. Isolates were selected in order to avoid those which could be epidemiologically related.

All isolates were serotyped according to the Kauffmann-White scheme using the traditional slide agglutination method, combined with a previously described PCR protocol to differentiate S. Typhimurium and S. 4,5,12:i-.

S. Enteritidis, S. Typhimurium and S. 4,5,12:i- were phage typed using the protocols and following the interpretative guidelines set out for S. Typhimurium and S. Enteritidis by the International Federation for Enteric Phage Typing (IFNPT, Laboratory of Enteric Pathogens, Health Protection Agency, Colindale, London, UK).

All isolates were processed both for genomic and plasmid DNA extraction. Total DNA was extracted by the classical boiling method, while the plasmid DNA was obtained from pelleted cells using the QIAprep Spin Miniprep Kit (Qiagen) and by applying the modifications to the protocols required for the isolation of high and low plasmid copy-number as described by the supplier.

ERIC-PCR typing. ERIC-PCR was performed as described by Fendri et al. Briefly, PCRs were prepared by adding 2 μl of DNA template to a reaction mix containing 12.5 μl of Go-Taq Green Master Mix (Promega) and 1 μM of each primer; the reaction volume was made up to 25 μl with autoclaved and filtered Milli Q water (Millipore, Billerica, MA, USA). PCRs were carried out using a My Cycler thermocycler (Bio-Rad). Primer sequences ERIC-1R (ATGAAGCTCGGGGATTCAC) and ERIC2 (AAGTAAAGTGAAGGGGTGAGG) were designed by Versalovic and co-authors. The ERIC-PCR profiles were obtained by electrophoresis of the different amplicons for 8 h at 40 V/cm, in 2% agarose Tris borate-EDTA (TBE) gel stained with Midori Green (Bulldog Bio). A 100 bp DNA Ladder H3 RTU (Nippon Genetics, Germany) was used as the PCR fragment size marker. Band profile similarities for the examined isolates were analyzed by Treecon software (Bioinformatics &
Evolutionary Genomics, Belgium) and Dice coefficients were calculated. A bootstrap resampling process (100 reiterations) applying the neighbor joining (NJ) method was used to assess the robustness of each individual phylogenetic node. In order to cluster the isolates according to the unweighted pair group method of averages (UPGMA), a cut-off of 90% was set.

Antimicrobial susceptibility testing.

Phenotypic assay. The isolates were subcultured onto tryptone agar slants at 4 °C, transferred to 15 ml of Mueller-Hinton broth and incubated at 37 °C overnight. Antimicrobial susceptibility was tested by using a commercial microdilution test (Sensititre® Salmonella plate – EUMVS2) against a panel of 14 antimicrobials: ampicillin (A), cefotaxime (FOT), ceftazidime (TAZ), chloramphenicol (Ch), ciprofloxacin (Cip), colistin (Col), florfenicol (F), gentamicin (GEN), kanamycin (KAN); nalidixic acid (Na), streptomycin (S), sulfamethoxazole (Su), tetracycline (T), and trimethoprim (Tr) according to the manufacturer’s recommendations. The results were assessed after 24 h of incubation at 37 °C. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the antimicrobial that completely inhibited visible growth. The results were thus analyzed according to the cut-offs set by EUCAST (www.eucast.org).

Genotypic assay. Bacterial DNA extracted from each isolate was examined for the presence of a panel of ARGs conferring resistance to quinolones (qnrA and qnrS), β-lactams (blaTEM and blashv), S (strA and strB), T (tet(A)) and sulphonamides (sulII and sulIII); the primer sequences are listed in Table 1 and the positive controls are listed in Table 2. Briefly, specific ARGs fragments were amplified by PCR essays, as previously described for ERIC-PCR except for the primer concentration (0.5 μM) and the thermal profile (5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at specific annealing temperature (Table 1), 30 s at 72 °C and a final extension of 5 min at 72 °C).

Plasmid molecular typing. DNA from Salmonella isolates was examined for the presence of the following incompatibility plasmid groups: Inc (A/C, H11, H12, I1, I2, L/M, N, K, B/O, W, P, T, U, R, Y, X1, X2, Y2, Z1, Z2).
HIB–M, FIB–M, FIA, FIB, FIC, FII, FIIS, FIIlk). Plasmid replicons of *Salmonella* isolates were detected and typed by PCR-based replicon typing (PBRT) as proposed by Villa et al.\(^4\), using the PBRT kit from DIATHEVA (DIATHEVA, Fano PU, Italy) according to the manufacturer's instructions.

**Screening for the presence of type II TA modules.** DNA of each isolate was examined by PCR for the presence of two *Salmonella* type II TAs: *vapB* and *ccdB*. Primers were designed to sequences *vapB*-f (TGAGYACCAGAGAACAACC), *vapB*-r (GAYGGAGCCTGATAACATTCC), *ccdB*-f (TTGTCTGAGAVACAGAGGAC) and *ccdB*-r (TATGCAYCACCGGGTAAAG) were designed based on conserved regions of TA genes using Clustal-XII software (http://www.clustal.org/) for multiple alignment, NetPrimer software (http://www.premierbiosoft.com/netprimer/index.html) for the primer design and BLAST analysis in order to verify primer specificity. PCR assays were performed as described above for ARGs detection with the annealing temperatures of 55°C for both *vapB* and *ccdB* genes.

**CcdB sequence analysis.** A fragment of 632 bp of the CcdB toxin gene containing its entire coding sequence (CDS) from four *Salmonella* isolates (S. 4, [5], 12;i−: 864_2013, S. Enteritidis 2011_2256/1, S. Enteritidis 2012_1058/2 and S. Typhimurium 149_2014) was amplified by PCR using the primers CCD_B-fw (5′-TACGACCAGACGAGAAC-3′) and CCD_B-rw (5′-CAATTCTGTAACCGGGCAA-3′), designed as previously described on the conserved region of textit{S}. Typhimurium strain SL1344 based on annotations and the sequence deposited in NCBI (entries NC_017720.1 (plasmid 1, pSLT) gene ID PSLT106). To achieve high fidelity gene amplifications, the Phusion® High-Fidelity DNA Polymerase (New England Biolabs Inc.) was used for fidelity gene amplifications, the Phusion® High-Fidelity DNA Polymerase (New England Biolabs Inc.) was used and the following thermal profile was applied: 30 s at 98°C, 25 cycles of 15 s at 98°C, 30 s at 53°C, 30 s at 72°C and a final extension of 5 min at 72°C. Amplicons were then bidirectionally sequenced, assembled and manually corrected using DNA Baser Sequence Assembly Software (Heracle BioSoft, Romania). The sequences were deposited at the European Nucleotide Archive (ENA) with accession numbers LN897324-LN897327. The assembled sequences were translated to proteins using the *Translate* tool (ExPASy–Bioinformatic Resources Portal, http://web.expasy.org/translate/). Protein sequences were aligned by means of the ClustalW2 tool (EMBL EBI, http://www.ebi.ac.uk/Tools/msa/clustalw2/) using the *E. coli* CcdB sequence as reference.

**Statistical analysis.** The Pearson Chi Square test was used to evaluate possible associations between the presence of FIIS plasmids and *Salmonella* serovars (S. Typhimurium and S. 4, [5], 12;i−: vs. other serovars). To verify whether a significant difference exists in the prevalence of FIIS plasmids among the investigated serovars, the \(z\)-test was used. \(P\) values less then 0.05 were considered significant. STATA 12 was used to analyze the data.

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Author Contributions
A.D.C. and C.L. conceived the experiments, conducted the experiments, analysed the results and wrote the manuscript, L.B. conceived the experiments, analysed the results and wrote the manuscript, D.C. and G.S. conducted the experiments, E.M.E. analysed the results and drawn Figure 1, G.C. and A.R. conceived the experiments and analysed the results. All authors reviewed the manuscript.

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