

## CHARACTERIZATION OF CLASS 1 INTEGRONS AND ANTIBIOTIC RESISTANCE GENES IN MULTIDRUG-RESISTANT *SALMONELLA ENTERICA* ISOLATES FROM FOODSTUFF AND RELATED SOURCES

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### ABSTRACT

In recent years, an increase in the occurrence of antimicrobial resistance among *Salmonella enterica* has been observed in several countries, which is worrisome because *S. enterica* is one of the most common causes of human gastroenteritis worldwide. The aim of this study was to characterize class 1 integrons and antibiotic resistance genotypes in *Salmonella enterica* isolates recovered from foodstuff and related sources. Nineteen multidrug-resistant (MDR) *Salmonella enterica* isolates were recovered. Higher resistance rates to tetracycline (90%), streptomycin (80%), sulfamethoxazole-trimethoprim (80%), ampicillin (60%) and nalidixic acid (70%) were related to the presence of the *tetA*, *aadA*, *sulI/sul2*, *bla<sub>TEM-1</sub>* genes, and a codon mutation at position 83 of the *gyrA* gene, respectively. Class 1 integrons harboring *aadA*, *bla<sub>TEM-1</sub>*, *sulI* or *dhfr1* genes were detected in nine (45%) *Salmonella enterica* strains belonging to serotypes Brandenburg, Panama, Agona, Mbandaka and Alachua. Finally, clonal dissemination of *S. Panama*, *S. Derby* and *S. Mbandaka* was confirmed by PFGE. Detection of clonally related MDR *Salmonella enterica* suggests that endemic serotypes can be supported by class 1 integron-borne gene cassettes and/or mutations in drug targets. Emergence and dissemination of multidrug-resistant *Salmonella enterica* can have a major public health impact in an environment where large-scale suppliers ship their products.

**Key words:** *Salmonella*, multidrug-resistant, class 1 integrons, foodstuff, Brazil

### INTRODUCTION

*Salmonella* is known as a cause of human gastrointestinal diseases following consumption of contaminated food. Pork meat and its derivatives are important sources of *Salmonella*, being overcome only by poultry products (11). The ubiquitous nature of *Salmonella* has created significant challenge for

animal husbandry, with antimicrobials being used either therapeutically or prophylactically, or as growth promotion (feed additives). Consequently, drug resistance in foodborne *Salmonella* species has been inevitable (15). In fact, the emergence of multidrug resistant (MDR) phenotypes have been described by several authors, especially among serotypes of *S. Typhimurium* (26), *S. Newport* (6), *Panama* (25) and

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Enteritidis (4). Moreover, several foodborne outbreaks caused by MDR *Salmonella enterica* have been reported in the literature (8, 17).

Multidrug resistance has been associated with classical mobile genetic elements (i.e., transposons and plasmids). However, in recent years, a novel group of DNA elements able to incorporate antimicrobial resistance genes cassettes by a site-specific recombination event have been identified in gram-negative bacteria. These elements are termed integrons (12), and up to date, at least nine classes of integrons have been described among clinical bacteria isolates, with class 1 being the most prevalent (22). Integrons have been described in human pathogens isolated from clinical samples (21), however, information regarding the presence of integrons among isolates from food and food processing environment is scarce.

This study aimed to characterize class 1 integrons and antibiotic resistance genes in multidrug-resistant *Salmonella enterica* isolates recovered from food and related sources.

## MATERIALS AND METHODS

### Bacterial strains and antibiotic susceptibility testing

Nineteen MDR *S. enterica* isolates, collected between 2003 and 2004, recovered from pork meat and products, pork feces, and pork slaughterhouse environment as well as poultry

were included in this study (Figure 1). Isolates were tested by disc diffusion and broth microdilution methods based on CLSI standards (7) for susceptibility to amikacin (Ak), ampicillin (Amp), cefotaxime (Ctx), ceftazidime (Caz), ciprofloxacin (Cip), chloramphenicol (Cm), enrofloxacin (Enr), sulfamethoxazole-trimethoprim (Sxt), tetracycline (Tc), gentamicin (Gm), kanamycin (Km), nalidixic acid (Nal), and streptomycin (Sm). *Escherichia coli* ATCC 25922 was used as a quality control strain. One strain of *S. Typhimurium* susceptible to all antibiotics was included in the study for control purposes.

### Detection of resistance genes

Chromosomal DNA of the isolates was extracted using the GenomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Biosciences, USA), and DNA amplification by PCR was carried out using specific primers (Table 1) to search for: i) *tetA*, *tetB*, and *tetG*; ii) *aadA* and *aadB*; iii) *dhfr1*; iv) *sul1* and *sul2*; v) *flo<sub>st</sub>*; vi) *bla<sub>TEM-1</sub>*; and vii) *qacEAI* genes, responsible for resistance to tetracyclines, aminoglycosides, trimethoprim, sulfonamides, phenicols, β-lactams, and quaternary ammonium compounds, respectively. The PCR reactions were carried out using primers and conditions previously reported (19, 20).

**Table 1.** Nucleotide sequences of the oligonucleotides used for amplification<sup>a</sup>

Gene target	Primer sequence (5 <sup>v</sup> to 3 <sup>v</sup> )	PCR product size (bp)	Annealing temperature (°C)
<i>aadA</i>	F: GTG GAT GGC GGC CTG AAG CC R: ATT GCC CAG TCG GCA GCG	526	62
<i>aadB</i>	F: TCC AGA ACC TTG ACC GAA C R: GCA AGA CCT CAA CCT TTT CC	700	54
<i>sull</i>	F: GCG CGG CGT GGG CTA CCT R: GAT TTC CGC GAC ACC GAG ACC AA	350	65
<i>sul2</i>	F: CGG CAT CGT CAA CAT AAC C R: GTG TGC GGA TGA AGT CAG	720	52
<i>tetA</i>	F: GTG AAA CCC AAC ATA CCC C R: GAA GGC AAG CAG GAT GTA G	890	53
<i>tetB</i>	F: CTC AGTATTCCAAGCCTTTG R: ACT CCC CTG AGC TTG AGG GG	415	54
<i>tetG</i>	F: GCT CGG TGG TAT CTC TGC TC R: AGC AAC AGA ATC GGG AAC AC	470	57
<i>flost</i>	F: ACC CGC CCT CTG GAT CAA GTC AAG R: CAA ATC AAC GGG CCA CGC TGT ATC	550	69
<i>blajEM</i>	F: CAG CGG TAA GAT CCT TGA GA R: ACT CCC CGT CGT GTA GAT AA	643	53
<i>qacEAI</i>	F: ATC GCA ATA GTT GGC GAA GT R: GCA AGG CGG AAA CCC GCG CC	800	59
<i>dhfr</i>	F: TGG CTG TTG GTT GGA CGC R: TCA CCT TCC GGC TCG ATG TC	256	56

### Detection and sequencing of class 1 integrons

Presence of class 1 integrons was evaluated using primers 5'CS-3'CS (14), and mapping of gene cassettes harbored in class 1 integrons was performed as previously described (16). Gene cassette arrays were confirmed by direct sequencing of the amplified products, and the resulting sequences were aligned and confirmed using the GenBank database and BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### Analyses of the *gyrA* quinolone resistance-determining region (QRDR)

Amplification and sequencing of the *gyrA* QRDR region of quinolone-resistant *S. enterica* was done according to a previously described PCR protocol (10). The PCR products were purified with the GFX PCR and DNA Gel Band Purification kit (Amersham Biosciences), and then sequenced using an ABI PRISM® 3100 Genetic Analyzer and the BigDye® Terminator v3.1 kit (Applied Biosystems, USA). The *gyrA* sequences of quinolone-resistant *S. enterica* isolates, and its putative protein counterpart, were compared to known *gyrA* sequences deposited in GenBank, using BLAST software.

### Macrorestriction analysis of chromosomal DNA using Pulsed Field Gel Electrophoresis (PFGE)

Isolates were compared by PFGE separation of *Xba*I-digested genomic DNA using the protocol proposed by the National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet) (5). Addition of 50µM thiourea in the electrophoresis buffer was included to prevent DNA degradation. Images were captured with EDAS 120 System (Kodak) under UV light 320nm. BioNumerics software (Applied Maths, St-Martens-Latem, Belgium) was used for dendrogram construction and clustering, based on the band-based Dice's similarity coefficient and using the unweighted pair group method using arithmetic averages (UPGMA). Band position tolerance was of 2.0% and optimization of 0.5%. Isolates were considered to belong to the same cluster when

similarity coefficient was  $\geq 90\%$ .

## RESULTS

### Antibiotic resistance phenotype and PFGE characterization

The 20 *Salmonella enterica* strains used in this study were distributed among 10 different serotypes including Panama (6), Mbandaka (4), Derby (3), Enteritidis (1), 1,4,[5],12:i:- (1), Agona (1), Alachua (1), Ouakam (1), Brandenburg (1) and Typhimurium (1, negative control). PFGE was performed on the 20 isolates revealing nine *Xba*I macrorestriction profiles named A to I (Figure 1). As shown in the dendrogram, three main clusters (G, H, I) were identified according to Dice similarity coefficient. Strains showing the PFGE patterns G (n=4), H (n=4) and I (n=4) were the most frequently isolated, being recovered from salami after curing and inspection table; ground pork meat; and pork feces; respectively. Different serotypes belonged to different PFGE patterns, with the exception of the PFGE pattern H, which clustered *S. Panama*, *S. Agona* and *S. Derby*. On the other hand, the PFGE pattern C clustered the serotypes *S. Typhimurium* and *S. 1,4,[5],12:i:-*.

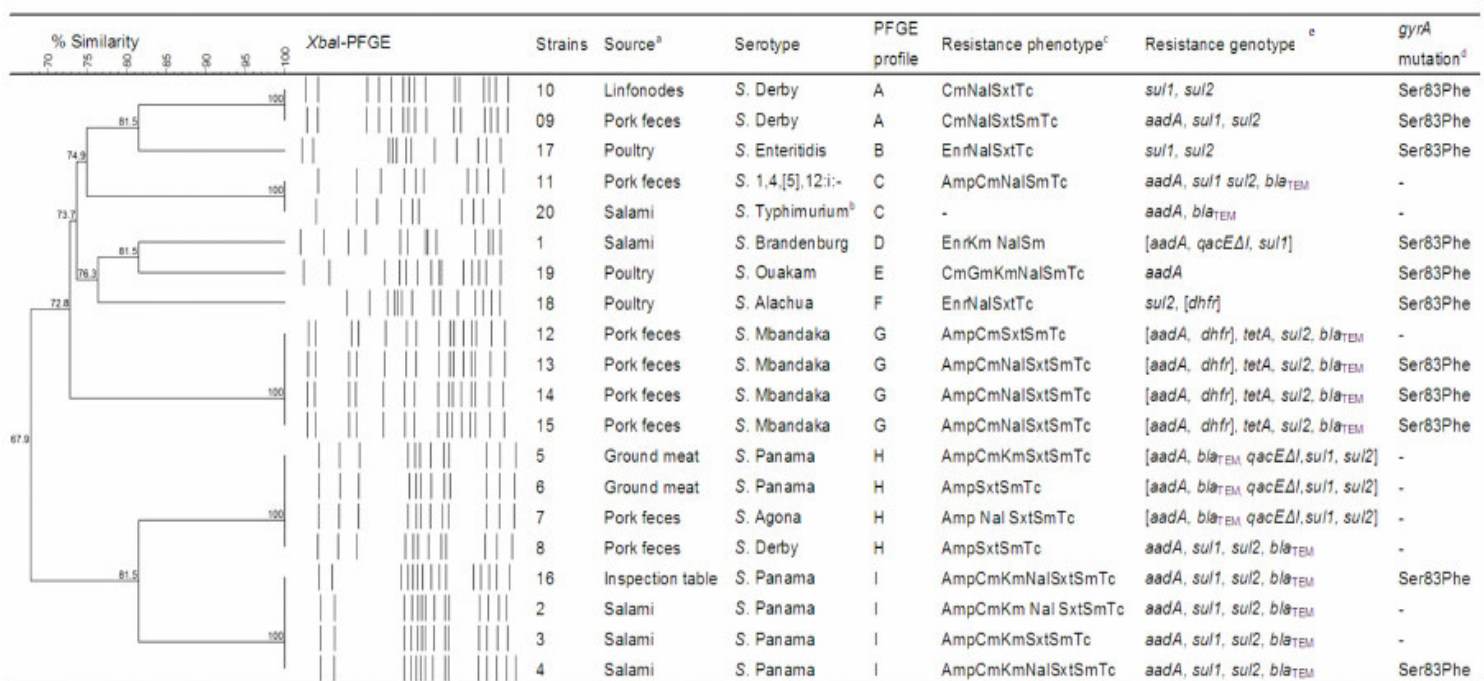
In Figure 1 is also summarized the antimicrobial resistance phenotypes of the 20 *Salmonella* isolates. All isolates were susceptible to amikacin, cefotaxime, ceftazidime, cefoxitin, and ciprofloxacin, but high resistance rates were observed to tetracycline (90%), streptomycin (80%), sulfamethoxazole/trimethoprim (80%), ampicillin (60%), nalidixic acid (70%) and chloramphenicol (60%). The rates of resistance to enrofloxacin, kanamycin and gentamicin were 15%, 35% and 5%, respectively. With the exception of the control strain of *S. Typhimurium*, all strains were multidrug-resistant exhibiting resistance to four or more agents of different antibiotic classes.

### Antibiotic resistance genotype

Six genes conferring resistance to five classes of antimicrobials — aminoglycosides (*aadA*), sulfonamides (*sulI*,

*sul2*), trimethoprim (*dhfr1*) tetracyclines (*tetA*) and  $\beta$ -lactams (*bla*<sub>TEM-1</sub>) — were identified by PCR. Additionally the gene *qacEΔI*, which confers resistance to compounds derived from quaternary ammonium, was also identified. Amongst the 17 isolates resistant to tetracycline, only *S. Mbandaka* amplified the *tetA* (Figure 1), whereas no isolates amplified the *tetB* or *tetG*. All streptomycin and sulfamethoxazole-trimethoprim-resistant isolates carried the *aadA* and *sul1* and/or *sul2*. The

*bla*<sub>TEM-1</sub> was found in all 13 ampicillin-resistant *S. enterica* isolates. Otherwise, *flo<sub>st</sub>* was not detected in any isolate, even in chloramphenicol resistant strains. The *aadA-sul1-sul2-bla*<sub>TEM-1</sub> resistance genotype was the most frequently observed (six isolates), being harbored by serotypes Panama, Derby and 1,4,[5],12:i:-. Finally, the *qacEΔI* gene was detected in serotypes Panama, Agona and Brandenburg harboring class 1 integrons (Figure 1).



**Figure 1.** PFGE of *XbaI*-digested DNA, serovars, susceptibility profiles, characterization of class 1 integrons, and genes associated with antibiotic resistance in twenty MDR *Salmonella enterica* strains recovered from foodstuff and related sources. This unweighted pair-group method with arithmetic mean dendrogram was generated using BioNumerics software (Applied Maths, St-Martens-Latem, Belgium) using the Dice coefficient. The scale above the dendrogram indicates percentage similarity. Using a 90% similarity cut-off point.

a No repeated samples were included; salami samples were taken after curing.

b Susceptible to all antibiotics tested.

c Amp (ampicillin), Cm (chloramphenicol), Enr (enrofloxacin), Gm (gentamicin), Km (kanamycin), Nal (nalidixic acid), Sxt (sulfamethoxazole/trimethoprim), Sm (streptomycin), Tc (tetracycline).

d Ser-83-Phe: Substitution of Serine for Phenylalanine in the codon 83 of the *gyrA* gene.

e [class 1 integron]; [*aadA, qacE\_I, sul1*], 1700 bp; [*dhfr*], 700 bp; [*aadA, dhfr*], 1700 bp; [*aadA, bla*<sub>TEM</sub>, *qacE\_I, sul1, sul2*], 2700 bp.

### Presence of class 1 integrons and their structural association with resistance genes

Class 1 integrons were detected in nine (45%) *Salmonella enterica* isolates belonging to serotypes Brandenburg, Panama, Agona, Mbandaka and Alachua. Integrons ranged in size from 0.7 Kb to 2.7 Kb. The *S. Brandenburg* and *S. Mbandaka* isolates carried class 1 integrons of 1700pb, whereas *S. Panama* (strain 5 and 6) and *S. Agona* (strain 7) harbored class 1 integrons of 2700pb. Curiously, *S. Alachua* isolate (strain 18) carried a 700 bp class 1 integron with a single *dhfr1* gene cassette, which appears to be the first report of class 1 integron in this serotype.

Class 1 integrons were sequenced for resistance gene mapping. Four different gene cassette arrays were found. Class 1 integrons from *S. Panama*, *S. Agona* (2700 bp) and *S. Brandenburg* (1700 bp) harbored the *aadA*, *qacEAI*, and *sulI* genes.

The observed size variation between integrons from these species was compatible with the insertion of a *bla<sub>TEM-1</sub>* cassette, located at the second position of the integron structure, in strains 3, 4, and 5. On the other hand, the *dhfr1* was the only gene detected in class 1 integron of *S. Alachua* (Figure 1), while integron of *S. Mbandaka* harbored *dhfr1* and *aadA* responsible for resistance to trimethoprim and streptomycin, respectively.

The *sul2* and *tetA* genes were not associated with class 1 integrons (Figure 1). On the other hand, *bla<sub>TEM-1</sub>* genes were located inside and outside the integron structure of ampicillin-resistant strains, showing wide distribution in the bacterial genome, and versatility of mobilization.

### Analyses of the *gyrA* quinolone resistance-determining region (QRDR)

Mutations in the *gyrA* gene were found in 11 (55%) *Salmonella enterica* isolates that showed resistance to nalidixic acid, of which three strains were resistant to enrofloxacin (Figure 1). The only mutation corresponded to codon at

position 83 (Ser → Phe). It is interesting to note that this mutation was found in different serotypes, including *S. Brandenburg*, *S. Panama*, *S. Derby*, *S. Mbandaka*, *S. Enteritidis*, *S. Alachua*, and *S. Ouakam* (Figure 1).

## DISCUSSION

The emergence of antimicrobial resistance in zoonotic bacteria has major public health implications. Data suggest that inadequate selection and abuse of antimicrobials use may lead to resistance in various bacteria, and drug resistance in foodborne bacterial enteric pathogens is an almost inevitable consequence of the use of antimicrobial drugs in food-producing animals (27). Thus, monitoring phenotypic and genotypic resistance to antibiotics in *Salmonella* species isolated from food-producing animals is important for the protection of human and animal health (24, 2).

In Brazil, high rates of bacterial resistance have been reported by the SENTRY and MYSTIC surveillance programs (23, 13), however these studies have been restricted to human bacterial pathogens from clinical origin. Very little is known about the incidence of resistance in food and food processing environment strains. In this study we characterized class 1 integrons, and antibiotic resistance in different serovars of multidrug-resistant *Salmonella enterica* strains isolated from pork products, pork feces, and pork slaughterhouse environment, as well as poultry.

The *Salmonella enterica* isolates included in this work belonged to serotypes Enteritidis, Typhimurium, 1,4,[5],12:i:-, Agona, Panama, and Mbandaka, which are among the 15 serotypes most prevalently isolated from clinical samples in Brazil (9). Additionally, the serotypes Agona and Derby which have been prevalent in Europe (1) were included.

Nine different PFGE patterns (named A - I) were identified among the isolates, with a genetic similarity ranging from 68% to 100%. It must be pointed out that no repeated samples of foodstuff and related sources were included. Thus,

clonal dissemination of *S. Panama*, *S. Derby* and *S. Mbandaka* was found. The presence of endemic *Salmonellae* in foodstuff and related sources could be considered an outcome of natural selection, due to the pressure induced by the repeated use of antibiotic in animal husbandry. In fact, phenotypic and genotypic resistance profiles of the isolates revealed the predominance of MDR phenotypes, exhibiting resistance to four or more agents of different antibiotic classes. In this regard, the higher resistance rates to tetracycline, streptomycin, sulfamethoxazole-trimethoprim and ampicillin were related to the presence of the *tetA*, *aadA*, *sulI/sul2*, and *bla<sub>TEM-1</sub>* genes, respectively. On the other hand, the higher resistance rate to nalidixic acid was due to codon mutation at position 83 in QRDR of the *gyrA*, resulting in the single change of amino acid residue, serine to phenylalanine. Most likely, this mutation lead to resistance to enrofloxacin, once three isolates carrying the Ser-83 to Phe mutation showed resistance to both nalidixic acid and enrofloxacin.

For some isolates, the phenotype and genotype relationship could not be assessed. In fact, *S. Typhimurium* harbored the *aadA* and *bla<sub>TEM-1</sub>*, but was susceptible to streptomycin and ampicillin, and *S. Brandenburg* harbored *sulI* and was susceptible to sulfamethoxazole plus trimethoprim. The lack of expression of these genes suggests that besides environment and genotype, gene expression is dependent upon upstream promoter (28). On the other hand, some isolates were resistant to chloramphenicol but no *flo<sub>st</sub>* could be detected. In this case other mechanisms could be responsible for the resistance phenotype observed. One mechanism could be the production of chloramphenicol acetyltransferases (encoded by *cat*) that has been recognized as an alternative mechanism to chloramphenicol resistance (3). Other studies should be conducted to check this hypothesis.

The wide dissemination of some resistance determinants (i.e., the *aadA*) observed in this study could be associated with the presence of class 1 integrons. Indeed, class 1 integrons were common in the endemic serotypes Panama and

Mbandaka, and differential size variation between these integrons was linked to the gene cassette arrays. Thus, flexibility of class 1 integrons structure will continue to contribute to the dissemination of gene cassettes in *Salmonella* species. In Brazil, class 1 integron carrying resistance gene cassettes has been documented in different serovars of *Salmonella* (18, 19). In this study, finding class 1 integrons in serovars Brandenburg, Panama, Mbandaka and Alachua, showing the dissemination of these genetic structures in serovars not previously reported.

## CONCLUSION

Data from this study indicates the dissemination of resistance genes in *S. enterica* isolated from foodstuff and related sources. The presence of clonally related MDR isolates suggests that endemic serotypes have been supported by class 1 integron-borne gene cassettes and/or mutations in drug targets. Moreover, emergence and dissemination of MDR *Salmonella enterica* in foodstuff and related sources has a major public health impact mainly for large-scale suppliers who ship their products both nationally and internationally. Thus, the risk of foodborne salmonellosis should be re-examined considering the increase of bacterial resistance.

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