Phenotypic and Genotypic Characterization of *Salmonella enterica* Recovered from Poultry Meat in Tunisia and Identification of New Genetic Traits

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Abstract

Thirty-seven *Salmonella enterica* isolates obtained from poultry meat in Tunisia were included in this study for characterization of antibiotic resistance mechanisms. High percentages of resistance were detected to ampicillin, sulfonamides, tetracycline, nalidixic acid, and streptomycin (32.4%–89.2%), and lower percentages to amoxicillin–clavulanic acid, kanamycin, amikacin, trimethoprim–sulfamethoxazol, and chloramphenicol (2.7%–18.9%). All strains showed susceptibility to ceftazidime, cefotaxime, gentamicin, and ciprofloxacin. Class 1 integrons were detected in 30% of *Salmonella* isolates, and four different gene cassette arrangements were detected, including genes implicated in resistance to aminoglycosides (*aadA1* and *aadA2*) and trimethoprim (*dfrA1*). Four different Pc variants (PcW, PcH1, PcH1_{TTN-10}, PcW_{TGN-10}) with inactive P2 have been found among these isolates. Integron-positive isolates were ascribed to eight different serotypes. A *Salmonella* Schwarzengrund isolate harbored a new class 1 integron containing the *qacH-dfrA1b-aadA1b-catB2* gene cassette arrangement, with the very unusual PcH1_{TTN-10} promoter, which has been registered in GenBank (accession no. HQ874651). Different plasmid replicon types were demonstrated among integron-positive isolates: IncI1 (8 isolates), IncN (8), IncP (2), IncFIB (2), and IncFII (2). Ten different pulsed-field gel electrophoresis profiles were detected among the 11 integron-positive isolates and 8 different sequence types were identified by multilocus sequence typing, one of them (registered as ST867) was new, detected in 3 *Salmonella* Zanzibar isolates. A high diversity of clones is observed among poultry *Salmonella* isolates and a high proportion of them show a multiresistant phenotype with very diverse mobile genetic structures that could be implicated in bacterial dissemination in different environments.

Key Words: Antibiotic resistance—Integrons—MLST—*Salmonella enterica*.

Introduction

FOOD-BORNE DISEASES caused by nontyphoidal *Salmonella enterica* serovars represent an important public health problem and an economic burden in many parts of the world (Miko et al. 2005, Alcaine et al. 2007). The main sources are foods of animal origin, such as eggs, milk, poultry, beef, and pork meat. In addition, fruits and vegetables have been incriminated as vehicles in *Salmonella* transmission.

In the last two decades, multidrug-resistant *S. enterica* isolates have been increasing and became a major health

hazard (Butaye et al. 2006, Alcaine et al. 2007). The use of antimicrobial agents in food-producing animals for different purposes, including infection treatment, disease prevention, and also growth promotion (allowed in some countries), has been a major factor in widespread dissemination of antimicrobial-resistant bacteria that could be transferred to humans through the food chain (Angulo et al. 2004).

Molecular typing techniques such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have been highly effective for epidemiological studies of

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S. enterica serotypes isolates from different origins (Hudson et al. 2001, Torpdahl et al. 2005, Seo et al. 2006).

Mobile genetic elements such as plasmids or transposons, which are able to disseminate antibiotic resistance genes by horizontal transfer, and integrons have been shown to play an important role in the evolution and dissemination of multi-drug resistance in Gram-negative bacteria (Liebert et al. 1999, Carattoli 2001). An integron is a site-specific recombination system that recognizes, captures, and expresses gene cassettes (Hall 1997, Fluit and Schmitz 1999). Several classes of integrons have been described, and the class 1 is the most prevalent one. Gene cassettes are composed of a single coding promoterless sequence and a recombination site *attC*, located at the 3' end of the gene, which is involved in the mobility of the gene cassettes. The expression of the gene cassettes included into the variable region of class 1 integrons is dependent of a Pc promoter located in the integrase *intI1* gene. A second promoter P2 is active in some of these integrons. Differences in excision and expression level of gene cassettes have been attributed to the variations detected in the sequences of Pc and P2 promoters (Lévesque et al. 1994, Jové et al. 2010).

The aim of this study was to establish the antimicrobial susceptibility patterns of *S. enterica* isolates from poultry meat recovered in Tunisia and to characterize the integron structures and the plasmid content of these isolates. Further, the genotypic relatedness of the integron-positive *S. enterica* isolates was deeply investigated.

Materials and Methods

Bacterial isolates

Thirty-seven isolates of *S. enterica* were included in this study. Eighteen isolates were obtained after analyzing 665 samples of poultry meat taken from two slaughterhouses during the period 2007–2008, and the remaining 19 isolates were selected from the collection of the microbiology laboratory of the Veterinary Research Institute of Tunisia (10 isolates recovered during 2002–2007 and 9 during 2009 from poultry meat of different abattoirs). *S. enterica* colonies were confirmed by API 20E (Bio-Mérieux, Marcy l'Etoile, France). Serotyping was performed in integron-positive isolates according to the Kauffmann-White scheme (Popoff and Minor 2001).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted on Mueller-Hinton agar plates (Difco, Sparks, MD) by the agar disk diffusion method according to CLSI criteria (CLSI 2009). The following antimicrobial agents were tested ($\mu\text{g}/\text{disk}$): ampicillin (10), amoxicillin (25), amoxicillin-clavulanic acid (20/10), ceftazidime (30), cefotaxime (30), gentamicin (10), kanamycin (30), streptomycin (10), amikacin (30), trimethoprim-sulfamethoxazol (1.25/23.75), tetracycline (30), nalidixic acid (30), ciprofloxacin (5), sulfonamides (200), and chloramphenicol (50). *Escherichia coli* ATCC 25922 was used as a control strain. The double disk test with cefotaxime or ceftazidime in the proximity to amoxicillin-clavulanic acid was used for the screening of extended-spectrum beta-lactamases (ESBL).

Polymerase chain reaction analysis and DNA purification

The presence of genes associated with resistance to ampicillin (*bla*_{PSE-1}, *bla*_{TEM}), chloramphenicol (*cmlA*, *floR*), tetracycline [*tet*(A), *tet*(B), *tet*(C), and *tet*(G)], quinolones (*qnrA*, *qnrB*, *qnrS*, and *qepA*), aminoglycosides [*aadA*, *aac*(6')-Ib, *aph*(3')-Ia, *strA*, and *strB*], and sulfonamides (*sul1*, *sul2*, and *sul3*) were detected by polymerase chain reaction (PCR) and DNA sequencing (Sáenz et al. 2004, Riaño et al. 2006, Rocha-Gracia et al. 2010). The study of *strA-strB* genes and its possible linkage to *sul2* gene was determined by PCR in all *sul2*-positive isolates (Vinué et al. 2010b, Soufi et al. 2011).

Study of the integron and characterization of the promoter of class 1 integron

The presence of the *intI1* and *intI2* genes and the 3' conserved segment (3'-CS) of class 1 integron (*qacED1 + sul1*) was studied by PCR in the 37 isolates. The variable regions of class 1 integrons were analyzed by PCR and sequencing (Sáenz et al. 2004). The characterization of nonclassic class 1 integrons lacking the 3'-CS region and associated with the *sul3* gene was carried out by a primer-walking strategy (Sáenz et al. 2010). The promoters (Pc), responsible for the expression of inserted gene cassettes, were studied by PCR and sequencing in the integron-positive *S. enterica* isolates (Vinué et al. 2010a).

Detection and classification of plasmids

Plasmids were classified according to their incompatibility group using the PCR replicon-typing method (Carattoli et al. 2005).

Clonal relationship and molecular typing in integron-positive *Salmonella* isolates

The clonal relationship among the integron-positive isolates was studied by PFGE (Sáenz et al. 2004) using 10 U of *SpeI* enzyme (New England Biolabs, Beverly, MA) for chromosomal DNA restriction. DNA patterns were interpreted as previously recommended (Tenover et al. 1995).

MLST was performed in the integron-positive *S. enterica* isolates to assign allelic profiles and a specific sequence type (ST). The *thrA*, *purE*, *sucA*, *hisD*, *aroC*, *hemD*, and *dnaN* housekeeping genes were studied by PCR and sequencing, and the obtained sequences were compared with those included in the website database (<http://mlst.ucc.ie/mlst/dbs/Senterica>).

Results and Discussion

Antimicrobial resistance phenotypes of *Salmonella* isolates

The susceptibility to 11 antimicrobial agents detected in the 37 *S. enterica* isolates is shown in Table 1. A high percentage of resistance was observed to ampicillin, sulfonamides, tetracycline, nalidixic acid, and streptomycin (32.4%–89.2%), as also reported in other studies (Chen et al. 2004, Zhao et al. 2005, Molla et al. 2007, Vo et al. 2010). Lower values of resistance were detected to amoxicillin-clavulanic acid, kanamycin, amikacin, trimethoprim-sulfamethoxazol, and chloramphenicol (2.7%–18.9%). All the studied isolates showed susceptibility to ceftazidime, cefotaxime, ciprofloxacin, or gentamicin.

TABLE 1. RESISTANCE TO 11 ANTIMICROBIAL AGENTS IN THE 37 *SALMONELLA ENTERICA* STRAINS ISOLATED FROM POULTRY MEAT

Antimicrobial agents	Number (%) of resistant strains		
	Integron-positive (n = 11)	Integron-negative (n = 26)	Total (n = 37)
Ampicillin	3 (27.3%)	9 (34.6%)	12 (32.4%)
Amoxicillin-clavulanic acid	1 (9.1%)	0	1 (2.7%)
Sulfonamides	11 (100%)	6 (23.1%)	17 (45.9%)
Streptomycin ^a	11 (100%)	22 (84.6%)	33 (89.2%)
Kanamycin	0	4 (15.4%)	4 (10.8%)
Amikacin	4 (36.4%)	0	4 (10.8%)
Nalidixic acid	8 (72.7%)	18 (69.2%)	26 (70.3%)
Chloramphenicol	5 (45.4%)	2 (7.7%)	7 (18.9%)
Tetracycline	6 (54.5%)	15 (57.7%)	21 (56.8%)
Trimethoprim-sulfamethoxazol	5 (45.4%)	1 (3.8%)	6 (16.2%)

^aStreptomycin resistance and intermediate categories according to CLSI standards are considered.

None of the isolates exhibited a positive ESBL phenotype. Twenty seven of the 37 *Salmonella* isolates (73%) showed a multiresistant phenotype (resistance to at least three different antimicrobial agent classes) that is frequent in *S. enterica*, as previously described (Chen et al. 2004, Randall et al. 2004, Molla et al. 2007, Vo et al. 2010). The detection of antimicrobial-resistant *Salmonella* in meat samples is of great concern, because they could be transmitted to humans through the food chain and could be implicated in human infections with a compromised treatment. The spread use of antimicrobial agents in therapy or prophylaxis or as growth promoters in poultry in Tunisia could be a factor for selection of resistant *Salmonella* that contaminate poultry meat.

Resistance genes in *Salmonella* isolates

Table 2 shows the resistance genes detected among the antimicrobial-resistant *S. enterica* isolates. Twelve ampicillin-

resistant *Salmonella* isolates were detected, and 11 of them (92%) harbored a *bla*_{TEM} gene. Twelve of the 17 sulfonamide-resistant isolates contained a *sul*-type gene (70%), 5 of them harbored more than one *sul* gene, and the following combinations were found: *sul1* + *sul3* (2), *sul2* + *sul3* (2), and *sul1* + *sul2* + *sul3* (1) (Table 2). Among the 33 streptomycin-resistant isolates, the *strA-strB* and *aadA* genes were found in 6 and 17 isolates, respectively, and both of them (*strA-strB* + *aadA*) were present in 6 isolates. The *strA-strB* gene pair and the *aadA* gene remain so far the most important mechanisms mediating streptomycin resistance in *S. enterica*. It is interesting to note that in two isolates, the *sul2* gene was found linked to *strA-strB* gene presenting the structure *repC-sul2-strA-strB*, which is widely disseminated (Vinué et al. 2010b, Yau et al. 2010, Soufi et al. 2011).

The *aph*(3')-Ia gene was detected in three of the four kanamycin-resistant isolates, and the *cmlA* gene in five of the seven chloramphenicol-resistant isolates. Among the 21 tetracycline-resistant isolates, 15 of them carried the *tet*(A) gene, but *tet*(B) and *tet*(C) were not detected. The *tet*(A) gene associated with tetracycline efflux pumps was reported to be the predominant one in *Salmonella* and *E. coli* isolates from livestock and food animals (Miko et al. 2005, Jouini et al. 2009, Soufi et al. 2009). None of the plasmid-mediated quinolone-resistance genes (*aac*(6')-Ib-cr, *qnrA*, *qnrB*, *qnrS*, or *qepA* genes) was detected among the 37 *S. enterica* isolates.

Integron detection and characterization of promoter of class 1 integrons

The presence of class 1 integrons was demonstrated in 11 of the 37 *Salmonella* isolates (30%), whereas no class 2 integrons were detected. Four different gene cassette arrangements were identified (Table 3). The genes implicated in resistance to aminoglycosides (*aadA1* and *aadA2*) and trimethoprim (*dfrA1*) were the most frequently found, as previously reported (Guerra et al. 2000, Krauland et al. 2009, Vo et al. 2010). Only two isolates harbored the classical *qacEΔ1-sul1* element at 3'-CS, and one of them was the only *Salmonella* Typhimurium strain (C4138) detected in this study. This strain C4138 showed the two integron patterns (*bla*_{PSE-1}, 1.0 kb and *aadA2*, 1.2 kb), and in addition, the presence of *floR* and *tet*(G) genes was demonstrated by PCR, indicative of the presence of

TABLE 2. GENES OF RESISTANCE DETECTED AMONG THE ANTIMICROBIAL-RESISTANT *SALMONELLA ENTERICA* STRAINS

Phenotype of resistance (number of strains)	Genes detected by PCR (number of strains)	
	Integron-positive strains (n = 11)	Integron-negative strains (n = 26)
Ampicillin (12)	<i>bla</i> _{TEM} (3)	<i>bla</i> _{TEM} (8)
Sulfonamides (17)	<i>sul1</i> (2)	<i>sul1</i> (0)
	<i>sul3</i> (4)	<i>sul3</i> (6)
	<i>sul1</i> + <i>sul3</i> (1)	<i>sul1</i> + <i>sul3</i> (1)
	<i>sul2</i> + <i>sul3</i> (2)	
	<i>sul1</i> + <i>sul2</i> + <i>sul3</i> (1)	
Streptomycin (33)	<i>strA-strB</i> (0)	<i>strA-strB</i> (6)
	<i>aadA</i> (8)	<i>aadA</i> (9)
	<i>strA-strB</i> + <i>aadA</i> (3)	<i>strA-strB</i> + <i>aadA</i> (3)
	<i>aph</i> (3')-Ia (0)	<i>aph</i> (3')-Ia (3)
Kanamycin (4)	<i>cmlA</i> (4)	<i>cmlA</i> (1)
Chloramphenicol (7)	<i>tet</i> (A) (5)	<i>tet</i> (A) (10)
Tetracycline (21)		

PCR, polymerase chain reaction.

TABLE 3. CLONAL RELATIONSHIP, INTEGRON STRUCTURES, AND PLASMID TYPES DETECTED IN THE 11 INTEGRON-POSITIVE *SALMONELLA ENTERICA* STRAINS RECOVERED FROM POULTRY MEAT

Strain	Serotype	Year	PFGE	MLST	Plasmid type	Class 1 integrons			
						<i>intI1</i>	Promoter	<i>qacEA1</i> + <i>sul1</i>	Arrangements included into the variable region
C4135	<i>Salmonella</i> Enteritidis	2007	P1	ST11	IncI1, IncN	+	PcW	–	<i>dfrA1-aadA1</i>
C3893	<i>Salmonella</i> Schwarzengrund	2007	P2	ST96	IncI1, IncN, IncFIB, IncFII	+	PcH1 _{TTN-10}	+	<i>qacH-dfrA1b-aadA1b-catB2</i>
C4136	<i>Salmonella</i> Saintpaul	2007	P3	ST27	IncI1, IncN, IncFIB, IncFII	+	PcW	–	<i>dfrA1-aadA1</i>
C4137	<i>Salmonella</i> Hadar	2008	P4	ST33	IncN	+	PcW _{TGN-10}	–	Unknown
C4138	<i>Salmonella</i> Typhimurium	2007	P5	ST19	IncI1, IncN	+	PcW _{TGN-10} ^a	+	<i>aadA2/bla_{PSE-1}</i>
C4139	<i>Salmonella</i> Zanzibar	2007	P6	ST867	IncI1, IncN	+	PcH1	–	<i>estX-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3-orf1-mef(B)Δ-IS26</i>
C4140	<i>Salmonella</i> Zanzibar	2009	P6	ST867	IncN	+	PcH1	–	<i>estX-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3-orf1-mef(B)Δ-IS26</i>
C4141	<i>Salmonella</i> Norwich	2009	P7	ST64	IncI1, IncP	+	PcH1	–	<i>estX-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3-orf1-mef(B)Δ-IS26</i>
C4142	<i>Salmonella</i> Virchow	2009	P8	ST16	IncI1	+	PcH1	–	<i>estX-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3-orf1-mef(B)Δ-IS26</i>
C4143	<i>Salmonella</i> Enteritidis	2009	P9	ST11	IncI1	+	PcW	–	<i>dfrA1-aadA1</i>
C4144	<i>Salmonella</i> Zanzibar	2009	P10	ST867	IncN, IncP	+	PcH1	–	Unknown

^aPcW_{TGN-10} variant was found in both integrons (*aadA2/bla_{PSE-1}*).
PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing.

Salmonella Genomic Island type 1 (Boyd et al. 2002, Carattoli et al. 2002, Miko et al. 2005, Vo et al. 2010). The other *qacEA1-sul1*-positive isolate is a *Salmonella* Schwarzengrund isolate (C3893) that harbored a new class 1 integron containing the *qacH-dfrA1b-aadA1b-catB2* gene cassette arrangement. This new integron, numbered as In150 according to the INTEGRALL database (<http://integrall.bio.ua.pt/?>), has been included in GenBank with the accession no. HQ874651. It is important to highlight that the usual *attC* site of the *aadA1b* gene cassette is missing, which could affect its mobilization. This fact has been observed in other sequences included in GenBank (e.g., accession nos. GQ422827, FM877485, or AY987853) only when *catB2* is the following gene cassette. Taking in account that the *attC* of *catB2* gene cassette is complete and functional for recombination, an *aadA1b::catB2* fusion of gene cassettes could be suggested.

Nine of the 11 integron-positive *Salmonella* isolates (82%) harbored a nonclassic class 1 integron (lacking the 3'-CS). The *dfrA1-aadA1* gene cassette arrangement was found in three isolates (two *Salmonella* Enteritidis and one *Salmonella* Saintpaul), and the *sul3* gene was associated with the long structure: *estX-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3-orf1-mef(B)Δ-IS26* in other four isolates (two *Salmonella* Zanzibar, one *Salmonella* Norwich, and one *Salmonella* Virchow). These structures were previously identified in both *Salmonella* and *E. coli* strains (Antunes et al. 2007, Sáenz et al. 2010, Vinué et al. 2010b, Soufi et al. 2011). All PCRs performed to detect the

gene cassettes behind the *intI1* gene were negative in the remaining nonclassic class 1 integron-positive isolates.

Gene cassettes of class 1 integrons may be differently expressed depending on the Pc (located within the integrase gene sequence) and P2 promoter variants. The Pc and P2 variants of the 12 detected gene arrangements were studied in the 11 integron-positive *Salmonella* isolates. An inactive P2 and four different Pc variants (PcW, PcH1, PcH1_{TTN-10}, PcW_{TGN-10}) have been found among these isolates (Table 3). Neither PcH2 nor PcS promoter variants were found. The PcW and PcH1 promoters have weak and intermediate strength, respectively, and they are the most prevalent Pc variants among the reported class 1 integrons in a previous *in silico* study (Jové et al. 2010). However, the presence in PcW of a TGN-10 motif that refers to an extended –10 hexamer (TGgTAAGCT), increases PcW efficiency near PcH2 one (Jové et al. 2010). In our study, the PcW_{TGN-10} variant was found in both integrons (*aadA2/bla_{PSE-1}*) of the *Salmonella* Typhimurium C4138 isolate. In addition, the PcH1_{TTN-10} variant, characterized by the substitution of a T instead of C in the second base upstream of the –10 hexamer (TTgTAAACT), slightly increases the PcH1 efficiency (Jové et al. 2010). This extremely rare PcH1_{TTN-10} promoter was found in the *Salmonella* Schwarzengrund strain that harbored the new *qacH-dfrA1b-aadA1b-catB2* integron. The involvement of integrons in multiresistance in *Salmonella* isolates has been extensively studied and reported by other

studies (Hall 1997, Guerra et al. 2000, Walker et al. 2001, Carattoli et al. 2002, Krauland et al. 2009).

Characterization of the plasmid types

Plasmids were studied in the 11 integron-positive *S. enterica* isolates and were classified according to their incompatibility groups. Five plasmid replicon types were identified and 7 of 11 isolates carried more than one different plasmid replicon (number of isolates): IncI1 (2), IncN (2), IncI1 + IncN (3), IncI1 + IncN + IncFIB + IncFII (2), IncI1 + IncP (1), and IncN + IncP (1). The IncI1, prevalent among the 11 strains of *Salmonella* analyzed, has been already associated with antibiotic resistance (Hopkins et al. 2006, Hradecka et al. 2008). Moreover, IncN are host-range plasmids that have significantly contributed in the global spread of resistance genes, including those conferring resistance to quinolones, carbapenems, aminoglycosides, and trimethoprim. The occurrence of IncI1 and IncN plasmids among *Salmonella* isolates of various serotypes highlights the high ability of these plasmids to disseminate. Indeed, both replicons are widespread in Enterobacteriaceae and have been recently associated with highly disseminated β -lactamase genes in *E. coli* and *Salmonella* from food animals (Hopkins et al. 2006, García-Fernández et al. 2008, Moodley and Guardabassi 2009, Rodríguez et al. 2009, Cloeckert et al. 2010).

Molecular typing of *S. enterica* isolates

All the 11 integron-positive *Salmonella* strains were analyzed by PFGE to study their genotypic relatedness. Digestion of *Salmonella* DNA with *SpeI* showed between 12 and 18 resolvable chromosome fragments ranging from ~48.5 to 728 kb. Ten distinct PFGE patterns (P1–P10) were generated among these strains and two of them showed an indistinguishable profile corresponding to *Salmonella* Zanzibar serotype (Table 3). PFGE analysis can separate different *Salmonella* serotypes into distinct clusters (Liebana et al. 2002, Peters et al. 2003). PFGE has been shown to be an efficient molecular tool for discriminating among *Salmonella* serovars recovered in our study and also in other studies carried out in Tunisia (Abbassi-Ghozzi et al. 2010).

Eight different STs were identified among our 11 integron-positive *S. enterica* isolates by MLST (Table 3). A new ST was detected in three *Salmonella* Zanzibar isolates and was registered under the name ST867. The ST11 was identified in two *Salmonella* Enteritidis isolates and the remaining isolates were ascribed to unique STs. It is worthy to note that our results are, in part, in agreement with those reported by Torpdahl et al. (2005). Indeed, they found that most isolates with the same serotype had the same ST and, most importantly, that ST11, ST27, ST16, and ST33 were specific to *Salmonella* Enteritidis, *Salmonella* Saintpaul, *Salmonella* Virchow, and *Salmonella* Hadar serotypes, respectively.

It is interesting to remark the high genetic heterogeneity of our strains, both by PFGE and MLST techniques. Strains belonging to the same ST did not always cluster in a single profile based on PFGE analysis; this is what we observed for the three strains that belong to a same ST867 but with different pulsotypes. Therefore, PFGE and MLST are complementary methods, appropriate for studies at distinct scales, for example, local epidemiology versus global population structure, respectively (Vimont et al. 2008).

Our findings support the hypothesis that the versatility of plasmids and the exchange of integrons and resistance genes by vertical and horizontal gene transfer may have contributed to the spread of these antimicrobial resistance traits. A systematic search for integrons and transposable elements could provide a useful genetic basis for multidrug-resistance spread in human and animal *Salmonella* isolates.

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Disclosure Statement

No competing financial interests exist.

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