Resistance-Gene Cassettes Associated With *Salmonella enterica* Genotypes

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Lab Med Spring 2015;46:90-96

DOI: 10.1309/LMFN8D17SOHQHGRP

Background: The epidemiology of salmonellosis is complex because of the diversity and different serotypes of *Salmonella enterica* (*S. enterica*) that occur in different reservoirs and geographic incidences.

Objectives: To determine the genotype distribution and resistance-gene content of 2 classes of integron among *S. enterica* isolates.

Methods: Thirty-six *S. enterica* species were isolated and tested for their serological distribution and the resistance-gene contents of 2 classes of integron, as well as for their genetic diversity, using the pulsed-field gel electrophoresis (PFGE) genotyping method.

Results: Serogroups E (36.1%) and D (30.5%) were dominant among the isolates. All of the isolates in serogroup D belonged to the serovar *enteritidis*. The *aad*A1 gene was found within all resistance-gene cassettes. We observed 4 common and 26 single pulsotypes among the isolates, which indicated a high degree of genetic diversity among the isolates. Using the PulseNet International standard protocol, it was found that these isolates were different from those reported previously in Iran.

Conclusions: The presence of a few common and new pulsotypes among the isolates suggests the emergence and spread of new clones of *S. enterica* in Iran.

Keywords: *Salmonella enterica;* serogrouping; integrons; genetic diversity; resistance, gene cassettes

Salmonella spp. are recognized as a prominent food-borne pathogen and an important cause of diarrheal disease among children less than 5 years old in developing and industrialized countries.[1] The widespread application of antimicrobial agents in clinical settings to treat infectious disease and the use of these agents in veterinary medicine are of great concern to public-health officials because use of these agents has led to development of multidrug-resistant (MDR) *Salmonella* strains.[2,3] Further, antibiotic-resistance genes can spread to other bacteria via mobile genetic elements, such as plasmids and transposons. Also, it has been shown that the resistance genes present on the plasmids and transposons of Gram-negative bacteria can be integrated into integron DNA elements. Integrons are the major reason for multidrug resistance in enteropathogens.[3] The occurrence of horizontal gene transfer between integrons carrying microorganisms and other enteric bacteria may result in the of emergence of integron-associated antibiotic resistance.[4,5] Several antibiotic resistance genes detected in Gram-negative bacteria are part of a gene cassette that is inserted in an integron.[6,7] Several classes

Abbreviations:

MDR, multidrug-resistant; PFGE, pulsed-field gel electrophoresis; TSI, triple sugar iron; SIM, sulfide-indole-motility; MR-VP, methyl red–Voges-Proskauer; PCR, polymerase chain reaction; CLSI, Clinical and Laboratory Standards Institute; dNTPs, deoxynucleotide triphosphates; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; UPGMA, unweighted pair group method with arithmetic average; *aad*A1, aminoglycoside adenyltransferase 1; CTs, common types; STs, single types; SXT, co-trimoxazol; TMP, trimetoprim; TE, tetracycline; NA, nalidixic acid; STR, streptomycin; AMP, ampicillin; CIP, ciprofloxacin; CHL, chloramphenicol

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of integrons based on the type of antibiotic-resistance elements have been identified by nucleotide sequence analysis.[7] Integron acquisition is considered to be the major cause of multiple antibiotic resistance in Gram-negative microorganisms, particularly in enteric bacteria.[6-8]

The epidemiology of salmonellosis is complicated because there are more than 2500 distinctive serotypes (*serovars*) with different reservoirs and geographic distributions.[9] In the past, differentiation among the individual strains of *Salmonella enterica* (*S. enterica)* for epidemiological purposes was limited to biochemical and serological identification. However, today, several DNA-base typing methods are available to investigate the interrelationship among Salmonella-species strains. One of the most reliable molecular-typing methods is pulsed-field gel electrophoresis (PFGE), which has been used worldwide to type *S. enterica* serotypes for epidemiological studies.[10,11] The establishment of the PulseNet International network has greatly helped in standardizing the typing methodology of *Salmonella* serovars and the normalization of PFGE patterns globally.[12,13]

Our study had 2 aims. First, we sought to determine the serotype distribution and the resistance-gene content of 2 classes of integrons among *Salmonella* strains isolated from diarrheal cases in Tehran, Iran. Second, we aimed to investigate the genetic diversity of those strains using the PFGE genotyping method.

Material and Methods

Bacterial Strains

We obtained 36 isolates of *S. enterica* from 3500 patients with acute diarrhea from a major hospital in Tehran, Iran. There was no evidence of outbreak; also, all fecal specimens were collected sporadically during a 12-month period from September 2009 through August 2010. All specimens were cultured on selective agar plates, including *Salmonella-Shigella* agar and XLD agar (Becton, Dickinson and Company, Franklin Lakes, NJ). We performed initial biochemical tests, including oxidase, triple sugar iron (TSI), sulfide-indole-motility (SIM), urea, methyl red–Voges-Proskauer (MR-VP), and L-lysine decarboxylase, to characterize the *S. enterica* isolates. The identity of isolates was confirmed using a genus-specific primer set, which amplifies the *inv*A region, as described elsewhere.[14] We performed serogrouping of the isolates via monospecific antisera provided by Bahar Afshan Institute of Research & Development, Tehran, Iran. The identity of the *S. enteric* serovar *enteritidis* isolates was confirmed by 2 multiplex–polymerase chain reaction (PCR) tests, as described previously.[15]

Table 1. Primers Used in This Study

Gene	Primers	Reference
invA	ACAGTGCTCGTTTACGACCTGAAT	
	AGACGACTGGTACTGATCGATAAT	14
in (gene cassette)	GGCATCCAAGCAGCAAGC	
	AAGCAGACTTGACCTGAT	18
int1	TGCGTGTAAATCATCGTCGT	
(class 1 integron)	CAAGGTTCTGGACAGTTGC	19
hep	CGGGATCCCGGACGGCATGCACGATTTGTA	
(class 2 integron)	GATGCCATCGCAAGTACGAG	20

Antimicrobial Susceptibility Test

We selected the antimicrobial agents according to the resistance-gene cassettes inside the variable regions of the integrons. Antimicrobial susceptibility testing was performed using the disc-diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[16] for ampicillin (10 µg), co-trimoxazole (25 µg) tetracycline (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), trimethoprim (5 µg), nalidixic acid (30 µg), and streptomycin (10 µg). We used *Escherichia coli* (*E. coli*)ATCC 25922 as a control in antimicrobial-susceptibility testing.

Integron Characterization and Sequencing of Resistance-Gene Cassettes

We extracted the total DNA via boiling[17] and used the resulting DNA as a template in PCR assays for the *invA* gene and class 1 and class 2 integrons, using the primers described in **Table 1**. PCR was performed in a reaction mixture with a total volume of 25 µl, containing 20 µl sterile water, 2.5 µl 10X Taq polymerase buffer, 0.3 µl deoxynucleotide triphosphates (dNTPs) (10 mmol l^{-1}), 1 U Taq DNA polymerase, and 30 pmol of each primer. Our PCR process was as follows: initial denaturation step at 94°C for 5 minutes, followed by 30 cycles consisting of denaturation (94°C for 1 minute), annealing (*invA*, 56°C; *int1*, 53°C; *in*, 56°C; and *hep*, 56°C, for 1 minute), and extension (72°C for 1 minute), followed by a final extension step at 72°C for 3 minutes. PCR products were purified

Table 2. Antibiotic Resistance Profile of *Salmonella enterica* Isolates

Antibiotic Resistance Profile	Isolates/Total Isolates, No. (%)[a]
SXT/TMP/TE/NA/STR/AMP/CIP	1 (2.8)
SXT/TMP/TE/NA/STR/AMP	1 (2.8)
TE/NA/STR/AMP/CIP	1 (2.8)
TE/STR/AMP/CIP	1 (2.8)
SXT/TMP/TE/NA/STR	5 (13.9)
SXT/TMP/TE/AMP/STR	1 (2.8)
SXT/TMP/TE/NA/AMP	1 (2.8)
SXT/TMP/TE/AMP	1 (2.8)
TE/NA/STR	2 (5.6)
STR/CIP/AMP	1 (2.8)
CHL/STR/AMP	1 (2.8)
STR/TE	3 (8.3)
NA	4 (11.1)
TE	2 (5.6)
No resistance	11 (30.6)

SXT, co-trimoxazol; TMP, trimetoprim; TE, tetracycline; NA, nalidixic acid; STR, streptomycin; AMP, ampicillin; CIP, ciprofloxacin; CHL, chloramphenicol.
[a]n = 36. Percentages may not total 100 because of rounding.

using the QIAquick Gel Extraction Kit (Qiagen N.V., Venlo, Netherlands) and direct sequencing of internal variable regions of class 1 and class 2 integrons was performed using the ABI 3730X capillary sequencer (Thermo Fisher Scientific Inc., Waltham, MA).

GenBank Accession Numbers

We entered 1 representative of each resistance-gene-cassette array inside class 1 integrons in the GenBank database. The assigned GenBank accession numbers are JX491633 and JX491634.

PFGE

We used PulseNet International standardized protocol for typing of *S. enterica* isolates.[21] Briefly, suspensions of bacteria from culture plates were adjusted to absorbance values of 0.8 to 1.0 at a wavelength of 610 nm in a cell-suspension buffer (100 mmol L^{-1} Tris; 100 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA]; pH, 8.0) from 400 µL, of which agarose plugs were prepared in plug molds using an equal volume of 1% SeaKem Gold agarose (Lonza Group Ltd., Basel, Switzerland) and 20 µL of proteinase K (20 mg/mL stock). We lysed cells in the agarose plugs by treating them with a lysis solution (50 mmol L^{-1} Tris; 50 mmol L^{-1} EDTA; pH, 8.0; 1% sarcosine; and 0.1 mg mL^{-1} of proteinase K) for 1 hour at 54°C. Six washing steps were performed, twice with sterile ultrapure water and 4 times with TE buffer (10 mmol L^{-1} Tris; 1 mmol L^{-1} EDTA; pH, 8.0). We applied 40 units of *Xba*I restriction enzyme to plugs in a freshly prepared buffer solution (33 mM Tris-acetate [pH, 7.9], 10 mM magnesium acetate, 66 mM potassium acetate, and 0.1 mg/mL bovine serum albumin [BSA]) and incubated the mixture at 37°C for 4 hours. *Xba*I-digested *S. enterica* serotype plugs of the Braenderup H9812 strain were used as DNA molecular-weight size markers. We performed electrophoresis using the CHEF Mapper XA System (Bio-Rad Laboratories Inc., Hercules, CA) at the level of 200 V at 14°C for 18 hours, with increasing pulse time from 2.16 seconds to 54.17 seconds. PFGE patterns were analyzed using GelCompar II software, version 4.0 (Applied Maths NV, Sint-Martens-Latem, Belgium); the patterns were compared by using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) clustering. We constructed a dendrogram using an optimization value of 0.5% and a position tolerance of 1%. Assignment of the types and interpretation of PFGE-generated patterns was performed according to the guidelines set down by Tenover and colleagues.[22]

Results

The prevalence of *S. enterica* was 1.0% (36/3500) among the patients with diarrhea. Serogrouping of the isolates showed the dominance of serogroups E (36.1%) and D (30.5%) among the isolates. The least prevalent was the isolates belonged to the serogroup A (2.8%). Further, serogroups B and C comprised 13.9% and 16.7% of the isolates, respectively. All of the isolates with D serogroup specificity belonged to the serovar *enteritidis*.

The foremost resistance profile was co-trimoxazol/trimethoprim/tetracycline/nalidixic acid/streptomycin (13.8%; **Table 2**). The most commonly observed type of antibiotic resistance was related to streptomycin, tetracycline, and nalidixic acid (47.2% for each), whereas only 1 isolate (2.8%) was resistant to chloramphenicol (**Table 2**).

PCR Assay and Sequencing Analysis of Integrons

Nine (25.0%) of the 36 isolates carried a class 1 integron (*int*$^+$) using primers int1-F and int1-R (5′-conserved region), with DNA bands of 900 bp (**Image 1**). Of those

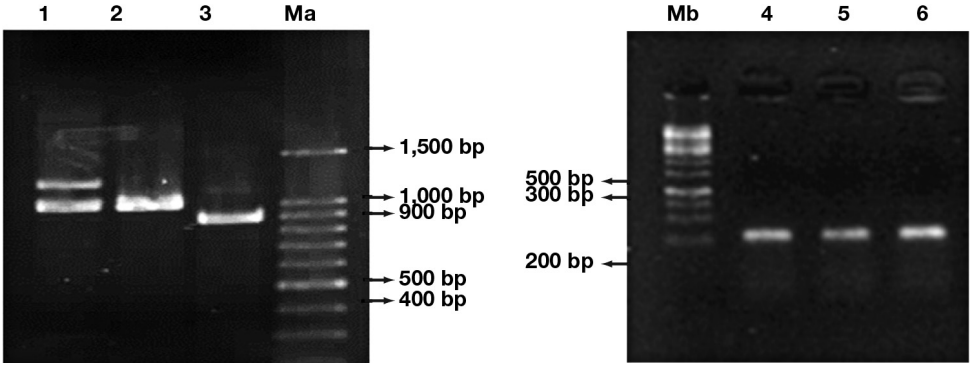


Image 1

Nine isolates of *Salmonella enterica*, as determined using primers int1-F and int1-R (5′-conserved region), with DNA bands of 900 bp (n = 36). **Lane 1**, internal variable rigion of class 1 integron with 2 gene cassettes of 1000 bp and 1100 bp, respectively. **Lane 2**, internal variable region of class 1 integron with 1000-bp gene cassettes. **Lane 3**, polymerase chain reaction (PCR) amplification of conserve integrase gene (*int1*) of class 1 integron (900 bp). **Lanes 4-6**, PCR amplification of *invA* gene (243 bp). Ma indicates 100-bp DNA size marker; Mb, 1-kb DNA size marker.

9 isolates, PCR amplification of the internal variable region using in-F and in-R primers produced 2 different amplification patterns of approximately 1000 bp in 5 isolates (55.5%) and 1000 bp plus 1100 bp in 3 (33.3%) of the *int*$^+$ isolates, respectively (**Image 1**). No product was obtained for 1 (11.1%) of the *int*$^+$ isolates, which indicated an empty integron with no resistance-gene cassette. Sequence analysis of the variable region of the class 1 integron indicated the presence of aminoglycoside adenyltransferase 1 (*aad*A1) and bla_{PSE1}-aminoglycoside adenyltransferase (bla_{PSE1}- *aad*A1) resistance-gene cassettes among the isolates, which corresponded to 1000-bp and 1100-bp PCR products, respectively. None of the 36 isolates harbored the class 2 integron, as determined using primers hep74-F and hep51-R. Six (66.7%) of the 9 integron-containing isolates belonged to serogroup C, whereas 3 (33.3%) belonged to serogroup B specificity. All isolates with the *aad*A1 resistance gene cassette belonged to serogroup C.

PFGE Analysis

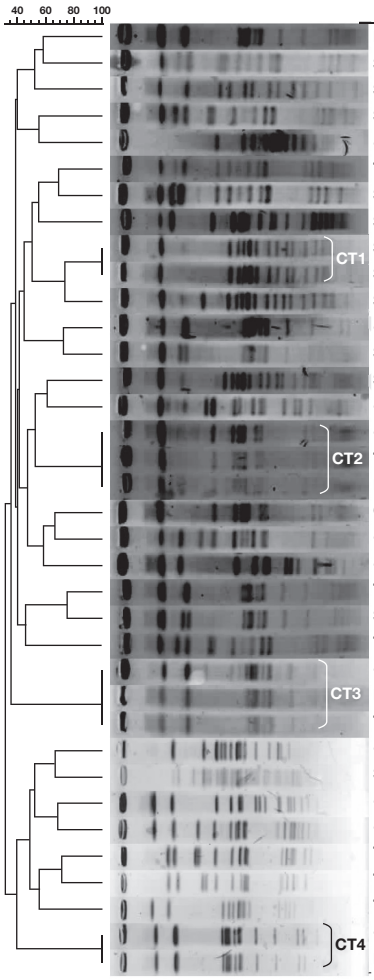
We compared the PFGE patterns using Dice coefficient and UPGMA analysis. In all, 4 common types (CTs), such as C1 through C4 with 100% identity and 26 single types (STs), were obtained among the isolates.

We further divided 25 Pulsotypes (A through Y) into 30 subtypes using the criteria presented by Tenover et al[22] (**Image 2**). Ten isolates were determined to be in the common-types category, with CT2 (pulsotype L) and CT3 (pulsotype R) each containing 3 isolates (8.3% of total isolates) and CT1 (pulsotype H1) and CT4 (pulsotype Y) each consisted of 2 isolates (5.5%). The remaining isolates were STs.

Discussion

In 2011, Ranjbar and colleagues[23] indicated that most *Salmonella* strains isolated between the years 2007 and 2008 in Iran were of serogroups D, C, and B. By contrast, we report that *S. enterica* isolates primarily belonged to serogroups D and E. This difference has occurred perhaps due to year and place of collection; it suggests the emergence of serogroup E and the persistence and adaptation of serogroup D of *S. enterica* over recent years in Tehran, Iran.

The highest resistance that we observed in our study was to streptomycin, tetracycline, and nalidixic acid (47.2% for each), whereas only 1 isolate (2.8%) was resistant to chloramphenicol. A high resistance rate of 61% to 68% against streptomycin and tetracycline has also been reported by other investigators[24,25] for *Salmonella* spp. However, the data on nalidixic acid and chloramphenicol resistance has been inconsistent.



Antimicrobial Resistance	Resistance Gene Cassette (Class 1 Integron)	Variable Region of Class 1 Integron	Serotype	Pulso Type
NA	-	-	D	A
STR/CIP/AMP	*aad*A1-*bla*PSE-1/*aad*A1	1,000 & 1,100 bp	B	B
SXT/TMP/TE/NA/STR	*aad*A1	1,000 bp	C	C
STR/TE	-	-	E	D
-	-	-	E	E
TE/STR/CIP/AMP	-	-	E	F1
SXT/TMP/TE/NA/STR/CIP/AMP	*aad*A1-*bla*PSE-1/*aad*A1	1,000 & 1,100 bp	C	F2
SXT/TMP/TE/AMP	-	-	E	G
SXT/TMP/TE/NA/STR	*aad*A1	1,000 bp	C	H1
SXT/TMP/TE/NA/STR	*aad*A1	1,000 bp	C	H1
SXT/TMP/TE/NA/STR	*aad*A1	1,000 bp	C	H2
-	-	-	D	I1
SXT/TMP/TE/NA/STR/AMP	-	-	D	I2
SXT/TMP/TE/NA/STR	*aad*A1	1,000 bp	C	J
-	-	-	E	K
-	-	-	E	L
TE	-	-	E	L
NA	-	-	E	L
CHL/STR/AMP	-	-	E	M
-	-	-	B	N
-	-	-	D	O
TE	-	-	D	P1
SXT/TMP/TE/NA/AMP	-	-	D	P2
TE/STR	-	-	D	Q
-	-	-	E	R
NA	-	-	D	R
TE/NA/STR	-	-	D	R
-	-	-	E	S
SXT/TMP/TE/AMP	-	-	E	T
-	-	-	A	U
-	*empty integron*	-	B	V
TE/STR	-	-	B	W1
TE/NA/STR/CIP/AMP	*aad*A1-*bla*PSE-1/*aad*A1	1,000 & 1,100 bp	B	W2
TE/NA/STR	-	-	E	X
-	-	-	D	Y
NA	-	-	D	Y

Image 2

Unweighted pair-group method with arithmetic mean (UPGMA) dendrogram of pulsotypes of XbaI digested of 36 *Salmonella enterica* isolates, with their antimicrobial resistance profile and class 1 integron content. Bolding indicates common types; NA, nalidixic acid; STR, streptomycin; CIP, ciprofloxacin; AMP, ampicillin; SXT, co-trimoxazol; TMP, trimetoprim; TE, tetracycline; CHL, chloramphenicol.

All resistance-gene cassettes bearing *int*$^{+}$ isolates harbored the *aad*A1 gene, which suggests the stability of this resistance gene among the *S. enterica* isolates. Some of us previously reported the predominance of the *aad*A resistance-gene family among enteropathogenic *E. coli* and *Shigella* spp., which is indicative of wide distribution of this subgroup of this resistance-gene family among members of the class *Enterobacteriaceae*.[26,27] Distribution of the class 1 integron has been reported to vary between 22.2% and 97% among different *S. enterica* serovars. However, a common feature of many reports has been the predominance of the *aad*A and *dfr*A resistance-gene families within the integrons.[28,29]

Molecular typing methods aid investigation of the relatedness of isolates by linking individual cases. However, final confirmation of isolate clonality relies on collaborative interpretation of laboratory and epidemiologic data, together with use of a comprehensive national typing database for long-term examinations. Results of the present study will add to our existing

database for intestinal bacteria, including *Vibrio cholerae*, *E. coli*, *Shigella* spp. and *S. enterica*, that has been developed over the past few years.[26,27,30] In this regard, we discovered a high level of genetic diversity among *Salmonella* isolates in Tehran province.

Pulsotype H (ie, H1 and H2) contained 3 isolates, all belonging to serotype C, which showed complete similarity in their antimicrobial resistance profiles and class 1 integron gene–cassette content (**Image 1**). This finding suggests that differences in pulsotype bands may occur due to point mutations or recombination events in the genome of the isolates. The L pulsotype with 3 isolates also showed an identical banding pattern; all 3 isolates belonged to serogroup E and were identified as integron-negative isolates with no resistance-gene cassettes. This may indicate that the isolates have acquired antimicrobial resistance factors through other genetic mechanisms, such as transposons or plasmids, rather than through integrons. The occurrence of different antimicrobial resistance patterns with an identical pulsotype and serogroup in integron-negative isolates was also observed in pulsotypes R and D. Ranjbar and colleagues[23] demonstrated that integron-positive *Salmonella* isolates belonged to serogroups C, D, and B. By contrast, in our study, we discovered integrons only among *Salmonella* serogroups B and C. The combined data from Ranjbar et al and ourselves suggests a change in the trend of integrons to specific serogroups in different consecutive years. Moreover, all isolates with *aad*A1 resistance-gene cassette belonged to serogroup C. The correlation of some resistance-gene cassettes with specific MDR *Salmonella* serovars was also reported by Molla and colleagues.[31]

Comparison of previous pulsotypes reported from Iran with those obtained in our study indicated considerable genomic diversity among the isolates examined in these studies. This finding indicates a rapid change in clonality and the emergence of new clones of *Salmonella* in Iran.[23]

In conclusion, the PulseNet International standard protocol used in the present study has helped us discover the diversities among Iranian strains of *S. enterica* in relation to their integron resistance-gene content. This finding has allowed us to add to a new national database on Iranian *Salmonella* isolates, which can be useful for national and international epidemiological studies of *S. enterica*.

Acknowledgments

We thank the Research Council of Tarbiat Modares University, Tehran, Iran, for supporting our project. LM

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