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Molecular Detection of Antimicrobial Resistance Gene Cassettes Associated with Class 2 Integron in *Salmonella* Serovars Isolated in Iran

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BR and SDS with the same contributions, designed the study, managed the literature searches, performed the analysis of data and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: *Salmonella* is an important food-borne pathogen in humans and a broad range of animals. Antimicrobial resistance in *Salmonella* spp. is a serious health problem in human and veterinary medicine worldwide. The aim of this study was to detect integrons, the natural recombination systems that can be transferred in companion with mobile genetic

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elements and play a major role in spreading antibiotic resistance genes in clinical isolates.
Place and Duration of Study: *Salmonella* clinical isolates were provided by a number of institutes and hospitals over the country through the years 2008-2009.
Methodology: Antimicrobial susceptibility testing and serotyping were carried out for eighty four epidemiologically unrelated clinical isolates of *Salmonella* serovars collected from different provinces of Iran through the years 2008-2009. PCR assays were carried out to detect *intI2* gene (integrase I attributed to class 2 integron) and internal variable regions (IVRs) of class 2 integron. These sequences were deposited in EMBL/GenBank database (www.ncbi.nlm.nih.gov).
Results: Eleven isolates (13.1%) which were resistant to at least 4 groups of antimicrobial agents were considered as MDR (multidrug resistant) *Salmonella* serovars. PCR assays detected *intI2* gene (integrase I attributed to class 2 integron) and internal variable regions (IVRs) of class 2 integron in Fourteen (16.7%) and eleven (78.6%) of *Salmonella* clinical isolates respectively. Analysis of the sequence data revealed 3 gene cassette arrays deposited in Genbank databases including the *dhfrA1* (0.75 kb), *dfrA14- lsp* (1 kb), *dhfrA1-sat2-aadA1* (3 kb) with three IVR distribution patterns. An artifact PCR product of 2 kb was reported in this study to be amplified together with IVRs of class 2 integrons which was associated with the *fhuE- ptsG* genes.
Conclusions: Presence of MDR *Salmonella* serovars demonstrates that antimicrobial selection pressure is widespread in our clinical settings. Detection of class 2 integron carrying gene cassettes which confer resistance to different classes of antibiotics such as aminoglycosides, and trimethoprim confirms that integron-mediated antimicrobial gene cassettes are prevalent in *Salmonella* serovars isolated in Iran.

Keywords: Salmonella; class 2 integron; gene cassettes; multidrug resistance (MDR).

ABBREVIATIONS

MDR: Multidrug Resistance.

1. INTRODUCTION

Salmonella is an important food-borne pathogen in humans and a broad range of animals. The spectrum of disease in humans comprises gastrointestinal infections and extra-intestinal infections such as bacteremia, central nervous system infections, bone and urinary tract infections [1].

Antimicrobial resistance in *Salmonella* spp. is a serious health problem in human and veterinary medicine worldwide. The intensive use of antibiotics in both human and veterinary medicine, as well as in agriculture, has caused bacteria to develop resistance mechanisms against therapeutic drugs which increases the morbidity, mortality and costs of treating infectious diseases [2,3].

Multiple antibiotic resistances are strongly associated with Integrons, the natural recombination systems that can be transferred in companion with mobile genetic elements and play a major role in spreading antibiotic resistance genes in clinical isolates [4]. Nine classes of integrons have been identified so far on the basis of nucleotide sequence of the integrase (*intI*) gene [5,6]. Their basic structure consists of an integrase encoding gene (*intI*), a recombination site (*attI*) and a promoter that controls the expression of gene cassettes (if

present). Gene cassettes comprise an open reading frame and a recombination site (*attC*), necessary for integration [6]. Class 2 integrons are part of the non replicative Tn7 transposon. They have a similar organization to class 1 integrons. The class 2 integrase (*IntI2*) has 46% amino acid identity to the class 1 integrase (*intI1*) [3].

High prevalence of multidrug resistance (MDR) in food-borne bacterial pathogens such as *Salmonella* prompted various studies on the antimicrobial drug resistance.

The present study was carried out to determine the antimicrobial susceptibility profile to detect MDR isolates and the prevalence of class 2 integrons as well as the distribution of integron- associated gene cassettes in clinical isolates of *Salmonella* spp. To achieve this purpose, isolates resistant to antibiotics commonly used in clinics were identified by antibiogram method and MIC determination. Integron-associated gene cassettes were detected by PCR assays in these isolates. Considering the potency of Integron to distribute among different isolates, this study was carried out to offer a pattern for rationale use of antibiotics.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates and Identification

The collection used for this study included 84 clinical isolates of *Salmonella* spp. recovered from stool (n= 69), blood (n= 6), bone marrow (n= 3), synovial fluid (n= 3), ascites (n= 1), abscess (n= 1), urine (n= 1) of patients (Table 1). These isolates were provided by a number of institutes and hospitals between 2008 and 2009 and were analyzed in Pasteur Institute of Iran. All isolates were identified by standard microbiological techniques as previously described [7,8]. The serogroup was checked with O antisera by slide agglutination method (Difco Laboratories, Detroit, MI).

Table 1. Information about 84 isolates of *Salmonella* serovars isolated from stool, blood, bone marrow, synovial fluid, abscess, ascites and urine

***Salmonella enterica* Serovars**	**Isolation source**							
	Stool	**Blood**	**Bone marrow**	**Synovial fluid**	**Abscess**	**Ascites**	**Urine**	**Total**
S. typhi	29	5	2	2	1	0	1	40
non-typhi serovars[1]	28	1	1	0	0	0	0	30
S. typhimurium	10	0	0	1	0	1	0	12
***S. paratyphi*A**	2	0	0	0	0	0	0	1
Total	69	6	3	3	1	1	1	84

[1]*non-typhi serovars are included S. enterica subsp. enterica serovar Enteritidis, Paratyphi B, Paratyphi C and Choleraesuis*

2.2 Antimicrobial Susceptibility Test

For each isolate, antibiogarm test was performed to evaluate the susceptibility to ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), trimethoprim (5 µg), sulfamethoxazole-trimethoprim (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), levofloxacin (5 µg), norfloxacin (5 µg), gatifloxacin (5 µg), moxifloxacin (5 µg), cefotaxime (30 µg), cefixime (5 µg), ceftriaxone (30 µg), cefepime

(30 µg), ceftazidime (30 µg), amikacin (30 µg), azithromycin (15 µg), spectinomycin (100 µg), gentamicin (10 µg), colistin-sulfate (10 µg), imipenem (10 µg) by disk diffusion on Muller–Hinton agar. Disks prepared by MAST Company (Mast Co, Merseyside, UK). Minimum Inhibitory Concentration (MIC) via broth microdilution method was carried out against all clinical isolates for the following antibiotics: ampicillin, chloramphenicol, streptomycin, nalidixic acid, ciprofloxacin, ceftazidime, trimethoprim, sulfamethoxazole and sulfamethoxazole-trimethoprim according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2009) [9]. The breakpoints for different antibiotics were referred in (Table 2). *E. coli* ATCC 25922 was used as a quality control organism in antimicrobial susceptibility test.

Table 2. Information about antimicrobial agents, break point, MIC range and antimicrobial resistance percentage for 84 samples of *Salmonella* serovars isolated from stool, blood, bone marrow, synovial fluid, abscess, urine, ascites

Antimicrobial agent[1]	Break-point for resistance (µg/ml)[2]	MIC range in isolates (µg/ml)	No. of isolates resistant to antimicrobial agents (%)				
			S. typhi (n=40)	non-typhiser ovars (n=30)	*S. typhimurium* (n=12)	*S. Paratyphi* A (n=2)	Total (%)
AMP	≥32	<4 - 2048	2 (5)	1 (3.3)	3 (25)	0 (0)	6 (7.1)
CAZ	≥16	<0.25 - 256	1 (2.5)	1 (3.3)	0 (0)	0 (0)	2(2.4)
CHL	≥32	<1 - >512	11 (27.5)	9 (30)	3 (25)	0 (0)	23 (27.4)
CIP	≥4	<0.01- 4	0 (0)	0 (0)	1 (8.3)	0 (0)	1 (1.2)
NAL	≥32	8 - >1024	28 (70)	20 (66.7)	5 (41.7)	1 (50)	54 (64.3)
STR	≥64	<1 - >512	10 (25)	8 (26.7)	6 (50)	1 (50)	25 (29.8)
TMP	≥4	<4- >2048	15 (37.5)	12 (40)	3 (25)	0 (0)	30 (35.7)
SXT	≥4/76	<4 - >2048	10 (25)	12 (40)	3 (25)	0(0)	25(29.8)
SMX	≥512	<16-8192	28 (70)	27 (90)	10(83.3)	2 (100)	67 (79.8)
No. of multi-drug resistant (MDR) isolates[3]			3(7.5)	5 (16.7)	3 (25)	0 (0)	11 (13.1)

[1]Abbreviation of mentioned antibiotics are AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; NAL, nalidexic acid; STR, streptomycin; TMP, trimethoprim ; SMX, sulfamethoxazole; SXT, sulfamethoxazole-trimethoprim

[2]Breakpoints were adopted from CLSI (Clinical and Laboratory Standards Institute)

[3]In this study isolates which were resistant to at least 4 groups of antimicrobial agents considered as MDR Salmonella serovars

2.3 Polymerase Chain Reaction (PCR)

DNA extractions were carried out as previously described [10]. All isolates were analyzed by PCR for the presence of the integrase gene of class 2 integrons (*intI2*) using primers introduced by Goldstein et al. [11]. The amplification program was performed by thermocycler (Eppendorf Mastercycler®, MA) and started with initial denaturation of 4min at 94˚C and programmed with 35 cycles of each: 1min at 94˚C, 30 s at 60˚C, 1 min at 72˚C. The program finished with the final extension of 10min at 72˚C. Subsequently, the variable regions of *intI2*-positive isolates were amplified using primers described by L'Abe'e-Lund and Sorum [12]. The touchdown PCR program to detect IVR2 introduced in this study was carried out as follows: initial denaturation at 94 ˚C for 4min and 10 cycles of 1min at 94˚C, 30 s at 52˚C (decreasing by 1.25˚C/cycle), 1min at 72˚C and 25 cycles of 1min at 94˚C, 30 s at 50˚C, 1min at 72˚C with the final extension of 10min at 72˚C.

2.4 DNA Sequencing

The PCR products were extracted from agarose gel and purified with the High Pure PCR Product Purification Kit (Roche, USA). According to the size of IVR amplified, one representative band of each group was sequenced using the ABI Capillary System (SEQLAB, Berlin, Germany). Sequences were compared with the GenBank sequences using online BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). Following this analysis, sequences were deposited in EMBL/GenBank database (www.ncbi.nlm.nih.gov).

3. RESULTS

3.1 Disk Diffusion and Minimum Inhibitory Concentration (MIC)

A total of 84 *Salmonella* isolates including *S.* Typhi (n = 40), non-Typhi serovars (n=30), *S*. Typhymurium (n=12), *S*. Paratyphi A (n = 2) were studied (Table 1). According to the Disk diffusion and Minimum inhibitory concentration (MIC), antimicrobial resistance patterns were as follow: 25 isolates (29.8%) were resistant to streptomycin, 25 isolates (29.8%) to sulfamethoxazole-trimethoprim, 30 isolates (35.7%) to trimethoprim, 23 isolates (27.4%) to chloramphenicol, 57 isolates (67.9%) to tetracycline, 6 isolates (7.1%) to ampicillin, 54 isolates (64.3%) to nalidexic acid, 1 isolate (1.2%) to ciprofloxacin, 6 isolates (7.2%) to cefotaxime, 8 isolates (9.5%) to cefexime, 6 isolates (7.2%) to ceftriaxon, 2 isolates (2.4%) to ceftazidime, 2 isolates (2.4%) to colistin-sulfate, 3 isolates (3.6%) to gentamicin, 24 isolates (28.6%) to spectinomycin, 5 isolates (5.9%) to azithromycin, 2 isolates (2.4%) to amikacin. All the isolates were sensitive to imipenem, ofloxacin, levofloxacin, norfloxacin, gatifloxacin, moxifloxacin. Of 84 isolates only 4 isolates (4.7%) were sensitive to the all of the tested antimicrobial agents. Multi-drug resistance was defined as resistance to at least 4 groups of antimicrobial agents. Of 84 isolates, 11 isolates (13.1%) were considered as MDR *Salmonella* serovars (Table 2). A similar result was obtained elsewhere by Rajaei et al. [13].

3.2 IntegronAnalysis and Characterization of Gene Cassettes

Fourteen isolates (16.7%) amplified *intI2* gene as previously reported [13] and eleven (78.6%) of *intI2*-positive isolates carried gene cassettes. Isolates harboring class 2 integron carried internal variable regions (IVRs) of 3 sizes, namely 750, 1000, 3000bp carried the *dhfrA1, dfrA14-lsp, dhfrA1-sat2-aadA1* gene cassettes arrays respectively. These IVRs were distributed among 84 *Salmonella* isolates with three patterns. An artifact PCR product of 2 kb were reported in this study to be amplified together with IVRs of class 2 integrons which was associated with the *fhuE-ptsG* genes(Fig.1 and Table 3).

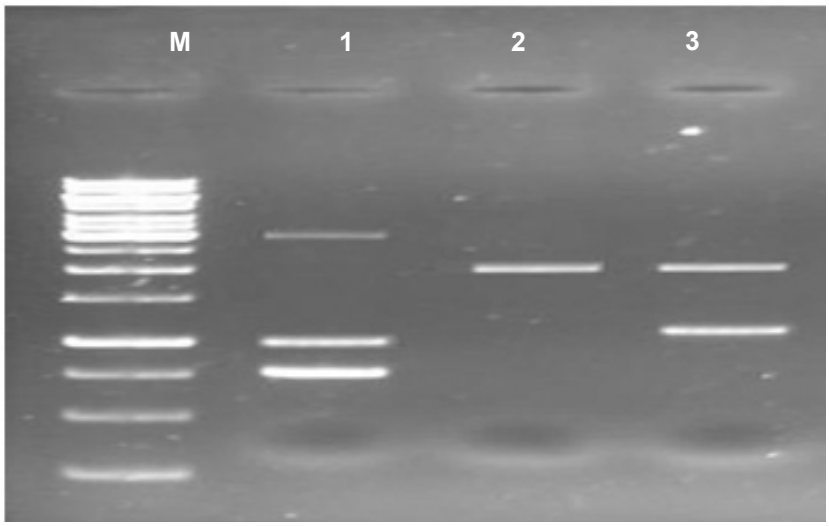

Fig. 1. **PCR amplification of Internal variable regions of class 2 integrons in *Salmonella* serovars**

Three distributions of Gene cassette arrays were shown as follows: Lane1 is the dhfrA1 (0.75 kb), dfrA14- lsp (1 kb), dhfrA1-sat2-aadA1 (3 kb); Lane 2 is the fhuE- ptsG (2 kb); Lane 3 is the dfrA14-lsp (1 kb), fhuE-psG(2 kb) ; Lane M is the 1 kb DNA ladder .

Table 3. Information about class 2 integrons regarding *intI*-positive, gene cassette region-positive and distribution of gene cassette arrays of *Salmonella* serovars

Serovars	No. of *intI2* positive isolates(%)[1]	No. of gene cassettes arrays positive isolates(%)[2]	Distribution of gene cassettes arrays(%)		
			dhfr1,dhfrA14-lsp,dhfrA1-sat1,aadA1	***dfrA14-lsp/ fhuE-ptsG***	***fhuE-ptsG***
S. Typhi (40)	5	4	1	3	0
Non-typhi serovars(30)	8	6	0	4	2
S. Typhimurium (12)	1	1	0	1	0
S. Paratyphi A(2)	0	0	0	0	0
Total(84)	14(16.7)	11(78.6)	1(9.1)	8(72.7)	2(18.2)

[1]*Indicates the number and percentage of intI1-posetive in Salmonella serovars*

[2]*Indicates the number and percentage of gene cassette internal region-positive isolates in total intI1-posetive isolates*

3.3 Nucleotide Sequence Accession Numbers

The nucleotide sequences of IVR in class 2 integrons were deposited in the NCBI GenBank sequence databases under the accession numbers [GenBank: JF264730, JN032741, JN032742, JF264731] which were attributed to the *dfrA14-lsp*, *dhfrA1*, *dhfrA1-sat2-aadA1*, *dfrA14-lsp* gene cassettes.

4. DISCUSSION

Exposure to antimicrobial compounds, impose bacterial species to recruit of exogenous genes in order to survive the environmental challenges. Integrons provide bacteria with a gene capture system perfectly adapted for the challenges of multiple-antibiotic treatment regimes [14].

In this study 84 clinical isolates of *Salmonella* spp. were studied to characterized integron-associated gene cassettes leading to multidrug resistance. Eleven isolates (13.1%) were found to be resistant to more than 4 groups of antimicrobial agents and defined as MDR in this study (Table 2). Fourteen isolates (16.7%) contained *intI2* gene and amplification of IVRs of *intI2*-positive isolates showed eleven (78.6%) isolates carried one or more gene cassette arrays. Of 14 isolates harbored class 2 integrons, one isolate considered as MDR. Careful studying is on progress to detect other classes of integrons in the rest ten MDR isolates. Detection of various classes of integrons in MDR isolates underlines the integron role in MDR distribution.

The content of IVRs in class 2 integrons and distributions of gene cassette arrays were as follow: one isolate carried three class 2 integrons of 0.75 kb, 1 kb, 3 kb sizes with the *dhfr1, dfrA14-lsp, dhfrA1-sat2-aadA1* gene cassettes respectively. This isolate belonged to the Typhi serovar of *Salmonella enterica*. Eight isolates harbored class 2 integron of 1kb size and an artifact PCR product of 2 kb which were associated with *dfrA14-lsp* and *fhuE-ptsG* respectively. Three of these isolates were Typhi serovars, one belonged to Typhimurium serovar and four related to non typhi serovars. Two other isolates exclusively amplified the artifact PCR product of *fhuE- ptsG* genes (Table 3).

The *aadA*1 product is aminoglycoside adenylyltransferase which confers resistance to streptomycin and spectinomycin [15]. The *dfrA14* and *dhfr1* products are dihydrofolate reductase which confers resistance to trimethoprim [16]. The *sat2* cassette confers resistance to streptothricin by encoding a streptothricin acetyltransferase [3]. The *lsp* product is lipoprotein signal peptidase [17].

Three isolates amplified *intI2* gene but not the IVR of the integron which were indicating (a) Some changes in the 3'-CS of the integron leading to the no band profile in these isolates according to the previous studies [18,19]. (b) An integron with a large number of cassettes called a super-integron, is too large to be amplified by conventional PCR techniques because of its considerable length [14,18]. (c) Some of the integrons harbor no gene cassettes in their IVR which are called In0. In this case the 5'-CS and the 3'-CS are not separated by the gene cassettes and they form empty structures [18,20].

The cassette array, *dfrA1-sat2-aadA1* detected in one of the *int2*-positive isolates, is usually found in Tn*7* transposons [14], confirming the association between class 2 integrons and the Tn*7* transposons.

The diversity of gene cassettes inserted in IVRs of class 2 integrons is much lower than class 1 integrons. This reduction in diversity is probably owing to a nonsense mutation in codon 179 (ochre 179) in the integrase gene of class 2 integrons, thereby yielding a truncated, non-functional protein [14].

Our results indicated that among the integron-positive isolates, the *dhfr* gene variations on class 2 integrons were prevalent and significant increase in resistance to trimethoprim and

trimethoprim–sulphamethoxazole was observed. Three types of cassette arrays detected in IVRs of class 2 integrons in this study, harbored *dhfr* gene alone or in companion with other genes. These data emphasizes the importance of class 2 integron in trimethoprim resistance distribution and this confirms the previous studies [11,14,18]. Trimethoprim is a broad-spectrum antimicrobial agent active against enteric pathogens, and trimethoprim alone or in combination with sulphamethoxazole has been commonly used to treat infection [18] but to date, fluoroquinolones, third-generation cephalosporins and imipenem are used as frontline therapeutic drugs to treat *Salmonella* infections.

In our study also, integron-positive isolates were resistant to different classes of antimicrobial agents but related resistance gene cassettes were not found to be harbored on the integron, implying the nonintegron sources of resistance to these antibiotics [18].

The gene cassettes arrays found among *Salmonella* spp. isolates in this study may also present in other clinical isolates of the Gram-negative bacteria, indicating the high potential of these structures to be transferred within microorganisms because of their transportation within plasmids or conjugative transposons. These results reveal that the same integron can disseminate in different bacterial species or genera, in different sample sources, and in different areas of the world [18].

In conclusion, Integron with the capacity of acquisition and dissemination of new antibiotic resistance genes, has the key role in distribution of multidrug resistance. Regarding this issue and to avoid distribution of multidrug resistance, the rationale use of antibiotics in clinics would be recommended.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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