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Multiple-Antibiotic Resistance in *Salmonella enterica* Serovars Isolated in Iran Harboring Class 1 Integrons

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Authors' contributions

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

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ABSTRACT

Aims: This research was carried out to detect the content and distribution of class 1 integrons in multidrug resistant *Salmonella* isolates.
Materials and Methods: Eighty four clinical isolates of *Salmonella* serovars were subjected to molecular detection of class 1 integrons following the antimicrobial susceptibility test using disk diffusion method and MIC determination.
Results: Eleven isolates (13.1%) which were resistant to at least 4 groups of antimicrobial agents

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considered as MDR (multidrug resistant) *Salmonella* serovars. The *intI1* gene and internal variable regions (IVRs) of class 1 integron were detected in 50 (59.5%) and 35 (70%) of *Salmonella* clinical isolates respectively. Analysis of the sequence data revealed four gene cassette arrays including the *dhfr7* (0.8 kb), *aadA1* (1kb), *blaP1* (1.2 kb), *dhfr1-aadA1* (1.6 kb) with eight IVR distribution patterns.

Conclusion: Detection of class 1 integron carrying gene cassettes which confer resistance to different classes of antibiotics such as aminoglycosides, ß-lactams and trimethoprim confirms that integron-mediated antimicrobial gene cassettes are prevalent in *Salmonella* serovars isolated in Iran.

Keywords: Class 1 integron; multidrug resistance; gene cassette array.

1. INTRODUCTION

Salmonella has been implicated in a wide variety of infections ranging from gastroenteritis to life-threatening typhoid fever and bacteremia [1]. Antimicrobial resistance in *Salmonella spp.* is a major health problem in human and veterinary medicine worldwide which increases the morbidity, mortality and costs of treating infectious diseases [2]. The threat of multiple resistance in bacterial strains and its wide dissemination are increased due to excessive antibiotic usage in both human and animal medicine [3]. The high prevalence of multidrug resistance (MDR) in foodborne bacterial pathogens such as *Salmonella* is an increasing problem and is not limited to specific countries or bacterial pathogens [4,5].

High levels of multidrug resistance are normally associated with mobile genetic elements that encode specific resistance genes. Among these genetic elements are the integrons, which are structures that can integrate and express resistance genes [6]. The capture of antimicrobial resistance genes by integrons and transmission of integrons together with mobile elements such as transposons, plasmids and genetic islands, underlies the rapid evolution of multiple drug resistance among clinical isolates of Gram-negative bacteria, including *Salmonella enterica* [7]. Five classes of integrons were introduced on the basis of nucleotide sequence of the integrase gene (*intI*) [8] but, to date, only those of class 1 and 2 have been reported in *S. enterica* [7]. Class 1 integrons are the most prevalent genetic system contributing in multiple antibiotic resistance in *Enterobacteriaceae* [9]. Class 1 integrons usually contain one or more resistance gene cassettes that constitute the internal variable region (IVR) flanked by two conserved segments (5'CS and 3'CS). 5'CS supplies the integrase gene (*intI*), the integration site (*attI*) and a strong promoter that ensures expression of the integrated gene cassettes. 3'CS carries additional resistance genes, such as the *qacEΔ1* and the *sul1* genesencoding low-level resistance to quaternary ammonium compounds and resistance to sulphonamids, respectively [7].

This research was carried out to characterize the antibiotic resistance profiles in clinical isolates of *Salmonella* serovars obtained in Iran through the years 2008 and 2009 and to detect the content and distribution of class 1 integrons in resistant isolates to study on the evolution of antibiotic resistance in human isolates of *Salmonella*. This is the first report of gene cassettes associated with class 1 integrons detected in *Salmonella* serovars in Iran.

2. METHODS

2.1 Bacterial Isolates

A total of 84 *Salmonella* isolates of clinical origin collected during 2008-2009. These isolates were 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). All isolates were identified by standard microbiological techniques as previously described [10]. The serogroup was checked with O antisera by the slide agglutination method (Difco Laboratories, Detroit, MI).

2.2 Antimicrobial Susceptibility Test

The antimicrobial susceptibility test was performed using the standard disk diffusion method on Muller-Hinton agar and the Minimum Inhibitory Concentration (MIC) via broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI) recommendations [11]. Disks prepared by MAST company (Mast Co, Merseyside, UK) were used to determine the susceptibility of isolates 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). The MICs of ampicillin, chloramphenicol, streptomycin, nalidixic acid, ciprofloxacin, ceftazidime, trimethoprim, sulfamethoxazole and sulfamethoxazole-trimethoprim were carried out against all clinical isolates. The breakpoints for different antibiotics were referred in Table1. *E. coli* ATCC 25922 was used as a quality control organism in antimicrobial susceptibility test.

2.3 Polymerase Chain Reaction (PCR)

DNA extractions were carried out using phenol-chloroform DNA extraction protocol [12]. All isolates were screened for detection of *intI1* gene with primers described by Goldstein et al. [13].The amplification program was performed by thermocycler (Eppendorf Mastercycler®, MA) and started with initial denaturation of 4min at 94ºC and programmed with 35 cycles of each: 1min at 94ºC, 30 s at 60ºC, 1min at 72ºC. The program finished with the final extension of 10min at 72ºC. The internal variable region of class 1 integrons were amplified using 5'-CS / 3'-CS primers as previously described by Martin et al. [6].The cycling program was as follow: initial denaturation at 94ºC for 4min and 35 cycles of 1min at 94ºC, 30 s at 56ºC, 1min at 72ºC, with the final extension of 10min at 72ºC.

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 the EMBL/GenBank database (www.ncbi.nlm.nih.gov).

3. RESULTS

3.1 Disk Diffusion Test

Of the 84 isolates only 4 isolates (4.7%) were sensitive to the all of the tested antimicrobial agents. The 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 nalidixic 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.

3.2 Minimum Inhibitory Concentration (MIC)

The antimicrobial resistance profiles in MIC assay were as follow: 25 isolates (29.8%) were resistant to streptomycin, 67 isolates (79.8%) to sulfamethoxazole, 30 isolates (35.7%) to trimethoprim, 25 isolates (29.8%) to sulfamethoxazole-trimethoprim, 23 isolates (27.4%) to chloramphenicol, 6 isolates (7.1%) to ampicillin, 54 isolates (64.3%) to nalidixic acid, 2 isolates (2.4%) to ceftazidime, 1 isolate (1.2%) to ciprofloxacin. Multi-drug resistance was defined as resistance to at least 4 groups of antimicrobial agents. Of the 84 isolates, 11 isolates (13.1%) were considered as MDR *Salmonella* serovars [14] (Table 1).

3.3 The Presence of Class 1 Integron via PCR

PCR assays of the 84 isolates presented that 50 isolates (59.5%) contained *intI1* gene and amplification of IVRs of *intI1* positive isolates showed that 35 isolates (70%) carried one or more gene cassette arrays. Isolates harboring class 1 integron were found to be carrying internal variable regions (IVRs) of 4 sizes, namely 800, 1000, 1200, 1600 which were associated with the *dhfr7*, *aadA1, blaP1, dhfr1-aadA1* gene cassettes respectively (Fig. 1). According to the

distribution of these IVRs, 8 profiles were designated (Table 2).

3.4 Nucleotide Sequence Accession Numbers

The nucleotide sequences of the *aadA1* gene, the *dhfr7* gene, the *blaP1* gene, the *dhfr1-aadA1* gene cassette and the *aadA1* gene in the class 1 integrons have been deposited in the NCBI GenBank sequence databases under the accession numbers HQ132374, HQ132376, HQ132377, HQ132378, HQ132375 respectively.

4. DISCUSSION

Limitless antibiotic administration generates selective pressure over bacterial species capable of incorporating new genetic material that may confer resistance to antimicrobial agents [15]. Integron as a mobile DNA element with the capacity of acquiring and disseminating gene cassettes, mainly antibiotic resistance genes by site-specific recombination, have the main role in MDR distribution leading to the limitation of treatment options for infections.

In this research 84 clinical isolates of *Salmonella spp.* were subjected to molecular investigations to detect integron-associated multidrug resistance. This is the first report of gene cassettes associated with class 1 integrons detected in *Salmonella* serovars in Iran. Fifty isolates (59.5%) contained *intI1* gene and amplification of IVRs of *intI1* positive isolates showed 35 isolates (70%) carried one or more gene cassette arrays.

In this study the contents of IVR and distributions of gene cassette arrays were as follow as illustrated in Table 2. Seventy isolates contained the *aadA1* gene (1kb). Two isolates harbored the *blaP1* gene (1.2kb). Four isolates carried the *dhfr7* gene (0.8kb). One isolate carried the *dhfr1-aadA1* gene cassette of 1.6kb on class 1 integron. Four isolates harbored two class 1 integrons with the *aadA1* (1kb) and *dhfr7* genes (0.8kb). Five isolates contained two class 1 integrons with the *aadA1* (1kb) and *blaP1* (1.2kb) genes. One isolate carried three class 1 integrons of 1kb, 1.2kb, 0.8kb sizes with the *aadA1*, *blaP1*, *dhfr7* genes, respectively. One isolate contained three class 1 integrons of 1kb, 1.2kb, 1.6kb sizes with the *aadA1*, *blaP1*, *dhfr1-aadA1* genes respectively (Table 2; Fig. 1). The *aadA*1 product is aminoglycoside adenylyltransferase which confers resistance to streptomycin and spectinomycin [16]. The *dhfr7* and *dhfr1* products are dihydrofolate reductase which confers resistance to trimethoprim [17]. The *blaP1* gene expresses a PSE-1/CARB-2 beta-lactamase which confers resistance to ampicillin [18].

It is noteworthy that the class 1 integrons were found in *Salmonella* isolates with a differing frequency and degree of spread among serovars (refer to Table 2). This result supports the previous studies [17].

Considering the abundance of different resistance gene cassettes, it appeared that cassettes encoding different aminoglycoside-modifying enzymes and dihydrofolate reductases were found in class 1 integrons most frequently in different studies [19,20]. This is in agreement with our analyses, where we found the *aadA*1 gene in 29 isolates and the *dhfr* gene in 11 isolates alone or in companion with other gene cassettes (Table 2).

Fifteen isolates amplified *intI1* 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 [20,21]. (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 [8,20]. (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 [20,22].

Sometimes the gene cassettes on the integrons may not be expressed or the isolate with resistance phenotype lacks the related gene cassette on the integrons. In this case non-integron elements involve in producing resistance [8,20]. This is in agreement with our results indicating the 4 isolates with the *dhfr*, 6 isolates with the *blaP1* and 13 isolates with the *aadA1* genes that lack the resistance phenotype of their related antibiotic. Furthermore, 24 trimethoprim resistant isolates, 3 ampicillin resistant isolates and 9 streptomycin resistant isolates did not carry the *dhfr, blaP1, aadA1* genes on the integron respectively.

Table 1. 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]	Breakpoint for resistance (µg/ml)[2]	MIC range in isolates (µg/ml)	No. of isolates resistant to antimicrobial agents (%)				
			S. Typhi (n=40)	non-typhi serovars (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, nalidixic 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 [14]

Table 2. Information about class 1 integrons regarding *intI*-positive, gene cassette region-positive and distribution of gene cassette arrays of *Salmonella* serovars

Serovars (No.)	No. of intI1-positive isolates (%)[1]	No. of gene cassette region-positive isolates (%)[2]	Distribution of gene cassette array (%)							
			aadA1	dhfr7	blaP1	dhfr1-aadA1	dhfr7, aadA1	aadA1, blaP1	aadA1, blaP1, dhfr7	dhfr1-aadA1, aadA1, blaP1
S. Typhi (40)	21	14	8	2	1	0	0	1	1	1
non-typhiserovars (30)	20	15	8	1	1	0	4	1	0	0
S. Typhimurium (12)	7	5	1	1	0	0	0	3	0	0
S. Paratyphi A (2)	2	1	0	0	0	1	0	0	0	0
Total (84)	50 (59.5)	35 (70)	17(48.5)	4 (11.4)	2 (5.7)	1 (2.8)	4 (11.4)	5 (14.2)	1 (2.8)	1 (2.8)

[1]indicates the number and percentage of intI1-posetive in Salmonellae serovars; [2] indicates the number and percentage of gene cassette internal region-positive isolates in total intI1-posetive isolates

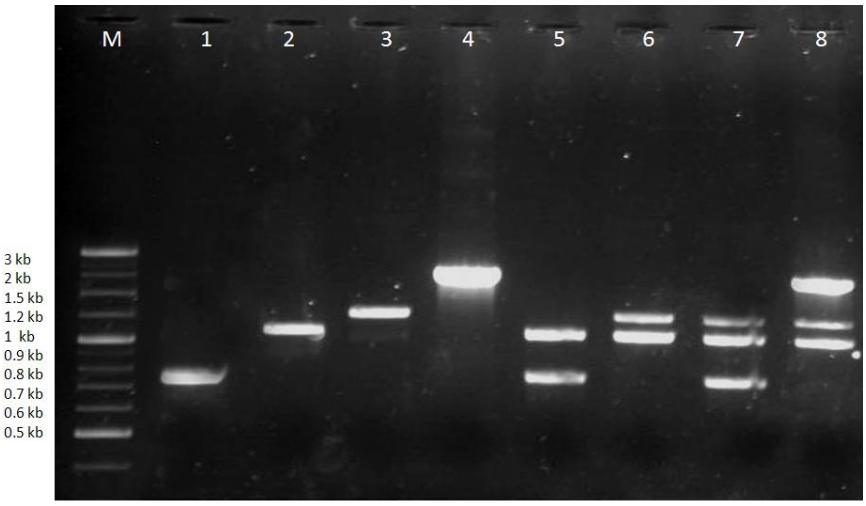

Fig. 1. PCR amplification of internal variable regions of class 1 integrons in S*almonella* serovars

Eight distributions of Gene cassette arrays were shown as follow: Lane 1 is the dhfr7 (0.8 kb); Lane 2 is the aadA1 (1kb); Lane 3 is the blaP1 (1.2 kb); Lane 4 is the dhfr7-aadA1 (1.6 kb); Lane 5 is the dhfr7 (0.8 kb), aadA1(1 kb); Lane 6 is the aadA1 (1 kb), blaP1 (1.2 kb); Lane 7 is the dhfr7 (0.8 kb) ,aadA1 (1 kb), blaP1 (1.2 kb); Lane 8 is the aadA1 (1 kb), blaP1 (1.2 kb), dhfr7-aadA1 (1.6 kb), Lane M is the 3kb DNA ladder

Our study indicates that all the MDR isolates harbored class 1 integron. This result highlights the integron role in MDR distribution. Otherwise some of the integron bearing isolates did not show the MDR profile.

Our data revealed that most class 1 integron-positive isolates are highly resistant to sulfonamides and trimethoprim. This data supports the previous studies and underline the importance of sulfamethoxazole-trimethoprim use in selecting integron-carrying *Salmonella* and emphasize the role of the hospital and other health care environments in the dissemination of such organisms [20].

Fluoroquinolones, third-generation cephalosporins and sulfamethoxazole-trimethoprim are considered to be frontline therapeutic drugs for treatment of *Salmonella* infections in hospitals. Also, carbapenems are the main class of drugs used for treatment of infections caused by MDR and extended-spectrum β-lactamase-producer Gram-negative bacteria such as *Salmonella* [10]. In *Salmonella* the gene cassettes located in IVRs of integron encode for older, although commonly used antibiotics, but the gene cassettes encoding resistance against the newest classes of antibiotics have not been detected yet [23]. Since the gene cassettes involving in the resistance of fluoroquinolones, third-generation cephalosporins and imipenem were not detected in this study to be harbored on class 1 integrons, therefore the distribution of these gene cassettes are much lower than those located on class 1 integrons and these drugs recommended to be used as frontline therapeutic drugs as before.

5. CONCLUSION

In conclusion, this research revealed the spread of integron-associated multidrug resistance in Iran. Our findings support the hypothesis that integron exchange represents a very efficient strategy for the acquisition of new antibiotic resistance genes. The presence of diverse integrons in *Salmonella* isolates accounts for the widespread multidrug resistance and would be important epidemiological tools to determine the distribution of MDR isolates following integon acquisition.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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