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...
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 *dhfrT* (0.8 kb), *aadA1* (1 kb), *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, β-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* genes encoding 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 94°C and programmed with 35 cycles of each: 1min at 94°C, 30 s at 60°C, 1min at 72°C. The program finished with the final extension of 10min at 72°C. 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 94°C for 4min and 35 cycles of 1min at 94°C, 30 s at 56°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 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 cefoxime, 8 isolates (9.5%) to ceftriaxone, 2 isolates (2.4%) to cefzidime, 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 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 aadA1 product is aminoglycoside adenylytransferase 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 aadA1 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 | Breakpoint for resistance (µg/ml) | 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)                 | 1 (3.3)              | 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(63.3)              | 2 (100)             | 67 (79.8) |
| No. of multi-drug resistant (MDR) isolates |   |                              | 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 *intI1*-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 | aadA1 | dhfr1, aadA1 | aadA1, blaP1 | dhfr1-aadA1, aadA1, blaP1 |
| S. Typhi (40)  | 21                                     | 14    | 8     | 2     | 1           | 0     | 0           | 1            | 1                      |
| non-typhiserovars (30) | 20                              | 15    | 8     | 1     | 1           | 0     | 0           | 4            | 1                      |
| S. Typhimurium (12) | 7                                  | 5     | 1     | 1     | 0           | 0     | 3           | 0            | 0                      |
| S. Paratyphi A (2) | 2                                  | 1     | 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) Indicates the number and percentage of *intI1*-positive in *Salmonella* serovars; 2) Indicates the number and percentage of gene cassette internal region-positive isolates in total *intI1*-positive isolates
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 integron acquisition.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.
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