

Study of Class 1 to 3 Integrons in *Salmonella* and Antimicrobial Resistance Pattern Isolated from Broiler Chicks

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Research Article

Abstract

High prevalent of multiple drug resistant (MDR) *Salmonella* is considered as a threat for human's health. Integrons are one of the most important factors that can contribute to the occurrence of MDR bacteria. The aim of this study was to determine the prevalence of class 1, 2 and 3 integrons among *Salmonella* strains isolated from broiler chicks. This study was performed on 100 *Salmonella* isolated strains, collected from male and female broiler chicks samples in southwest of Iran. The prevalence of class 1-3 integrons were verified using specific primers by multiplex PCR assay. Also antimicrobial susceptibility testing by disk diffusion method was performed for each isolate. Screening of *Salmonella* isolates revealed the prevalence of class 1, 2 and 3 integrons (50%), (28%) and (48%), respectively. Based on the results of this study significant correlations were between MDR and integrons, this is a serious problem in human and veterinary medicine. According to these results Ampicillin was the most resistant antibiotics against *Salmonella* isolated strains. The resistance to Gentamicin and Tetracycline and Chloramphenicol has increased in the presence of integrons. The presence of all three classes of integrons and its direct connection with the MDR in *Salmonella* is concerned.

Keywords: Broiler chicks; Integrons; Iran; Multiplex PCR; *Salmonella*.

1. Introduction

Salmonella is a facultative intracellular pathogen that causes a variety of infectious diseases such as gastroenteritis. It is one of the serious health problems worldwide and causes global morbidity and mortality [1]. Human salmonellosis is one of the most common and economically important zoonotic diseases and can affect all species of domestic animals, especially

pigs and poultry. High prevalent of multidrug resistance (MDR) *Salmonella* are considered as a threat for human's health [2]. The genus of *Salmonella* belongs to the Enterobacteriaceae family and includes more than 2500 different serotypes or serovars [3]. These bacteria are rod-shaped, gram-negative, mesophilic, non-spore-forming, predominantly motile and peritrichous flagella [2]. *Salmonella* is an important cause of bacterial food borne disease in industrialized countries and it is responsible for significant economic losses and serious health problems [4]. Although most *Salmonella* infection is limited to uncomplicated gastroenteritis that seldom requires antimicrobial treatment and severity of gastroenteritis and instead may result in prolonged fecal excretion and emergence of resistant strains [5]. Antibiotic resistance of *Salmonella* is a major threat to human health and veterinary worldwide [6,7]. Currently, the indiscriminate use and misuse of antibiotics has facilitated the emergence of resistance in many *Salmonella* serovars [8]. Virulence factors and resistance may be present on chromosomes, plasmids, transposons, integrons or phage located and they are able to integrate and express genes coding for antibiotic resistance [1].

Integrons are genetic elements that lead to various combinations of MDR pattern [9]. Horizontal transfers of integrons are an important factor of MDR in gram-negative bacteria [10,11]. Because of integrons are not capable of horizontal gene transfer themselves gene cassettes are necessary for combination in integron and often associated with transposons or plasmids. Over 80 gene cassettes and 8 classes of integrons have been characterized to date [12,13]. Class 1 and 2 integrons were found to be the most common and are referred to as resistance integrons but class 3 integrons are rare [14]. Classes 1 integrons are the most abundant type of integrons which are important for the creation and transfer of antibiotic resistance [1]. All integrons characterized

are composed of three key elements necessary: a gene encoding an integrase belonging to the tyrosine-recombinase family (int), a primary recombination site (attI) and promoter (Pc) [15]. The aim of this study was to prevalence of class 1, 2 and 3 integrons of *Salmonella* that isolated from broiler chicks and their relationship with MDR and also associations between integrons with drug resistances.

2. Materials and Methods

2.1 Bacterial isolation and identification

The number of 100 samples was collected randomly from male and female broiler chicks in southwest of Iran. After culturing and isolating bacteria, genomic DNA for the polymerase chain reaction (PCR) assays was performed by the boiling method [1].

2.2 PCR

The presence of class 1-3 integrons in *Salmonella* isolates were tested by multiplex PCR using specific primers for integrases genes of the integron, intI1, intI2, and intI3 introduced by Kargar et al. [11]. Each 50 μ L reaction mixtures contained 10x reaction buffer, 0.5 μ L dNTP mixture and 1.5 μ L $MgCl_2$ along with 0.5 unit TaqDNA polymerase, 0.75 μ L of each primer (class 1, 2 and 3 integrons) and 2.5 μ L of template DNA. For negative control a tube containing PCR reaction without any DNA template was used [11].

To identify genes related to the genera and species of enteritidis in *Salmonella* SefA primers were used. Primer sequences and PCR conditions were shown in Table 1. PCR amplification was performed in a 25 μ L reaction volume containing 1 μ g of genomic DNA, 1 μ M of each primer (forward and reverse of Sef A), 2 mM $MgCl_2$, 200 μ MdNTP, 2.5 μ L of 10x PCR buffer and 1 unit of Taq DNA polymerase (CinnaGen Co, Iran). The PCR products were analyzed by electrophoresis through 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed [16].

2.3 Antimicrobial susceptibility testing

Antibiotic resistance test were performed using standard Bauer-Kirby disk diffusion method for all 100 samples. Antimicrobial agents tested were Trimethoprim-sulfamethoxazole (SXT=1.25/23.75 μ g), Gentamicin (GM=10 μ g), Ciprofloxacin (CP=5 μ g), Nalidixic acid (NA=30 μ g), Chloramphenicol (C=30 μ g), Tetracycline(tet=30 μ g), Clindamycin (DA=2 μ g), Ampicillin (AMP=10 μ g), Cephalexin (CL=30 μ g) (Padtanteb, Tehran, Iran). Diameters of the inhibition zones were interpreted according to the Clinical and Laboratory Standards Institute (CLSI).

2.4 Statistical analysis

All data were analyzed by using MS Excel 2010 and SPSS software (Version 20. SPSS Inc., USA). At first, the frequency of resistance for each antibiotic (include 3 group: sensitive, semi-sensitive and resistance) was calculate and then the significant difference between this 3 groups at each integron for antibiotics was calculated using Chi-square and Fisher's exact tests.

3. Results

The result of multiplex-PCR results was showed in Figure 1, the size bands for integron class 1, 2 and 3 was 483 bp, 788 bp and 588 bp, respectively.

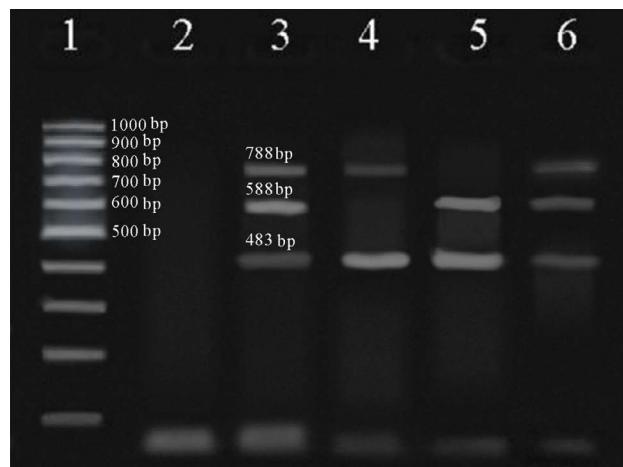


Figure 1. Multiplex-PCR results of three integrons. 1: marker (100 bp); 2: Negative control.

Table 1. Primers and PCR conditions used in this study.

Primer name	Primer sequence	Observed size (bp)	PCR conditions
<i>IntI 1-F</i>	ACATGCGTGTAAATCATCGTC	483	5 min at 94°C 32 cycles of 1 min at 94°C 1 min at 59°C 1 min at 72°C 10 min at 72°C
<i>IntI 1-R</i>	GGTCAAGGATCTGGATTTCG	788	
<i>IntI 2-F</i>	CACGGATATGCGACAAAAAGG	588	
<i>IntI 2-R</i>	ACATGCGTGTAAATCATC GTC		
<i>IntI 3-F</i>	TGTTCTTGTATCGGCAGGTG		
<i>IntI 3-R</i>	AGTGGGTGGCGAATGAGTG		
<i>Sef A-F</i>	TGCTATTTGCCCTGTACACTCG	214	5 min at 94°C 32 cycles of 1 min at 94°C 1 min at 63°C 1 min at 72°C 10 min at 72°C
<i>Sef A-R</i>	TTCGGGGAGACTATACCTACAG		

The PCR results in Figure 2, showed that bands 214 bp of the Sef A primers. Specific PCR analysis showed twenty-nine (29%) out of 100 isolates were species of *S. enteritidis*.

Based on the results of multiplex-PCR, the frequency of integrons 1, 2 and 3 in all of isolated samples were estimated as 50, 28 and 48 percent, respectively. The prevalence 3 groups integrin in *S. enteritidis* were 20, 12 and 30 percent, respectively (Graph 1).

The present of simultaneous occurrence of integrons 1 and 2 were 24%, 1 and 3 were 36%, 2 and 3 were 22% and present of simultaneous occurrence of integrons 1, 2 and 3 were 18% (Graph 2).

The antibiotic resistance results indicated that all isolates as well as samples of positive integrons was sensitive to Ciprofloxacin and Trimethoprim-sulfamethoxazole. In addition in all isolates 90% were resistant Clindamycin, 85% were resistant to Ampicillin, 57% resistant to Cephalexin, 5% resistant to Gentamicin and Tetracycline. However, the interpretation of the results show 100% of strains that have class 1, 2 and 3 integrons were resistant to Cephalexin, Clindamycin and Ampicillin.

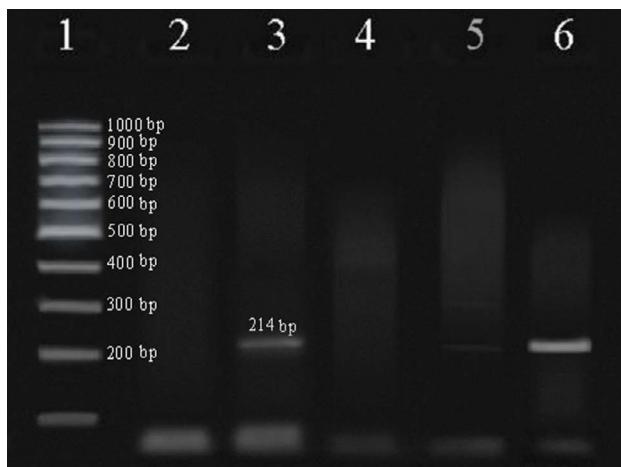
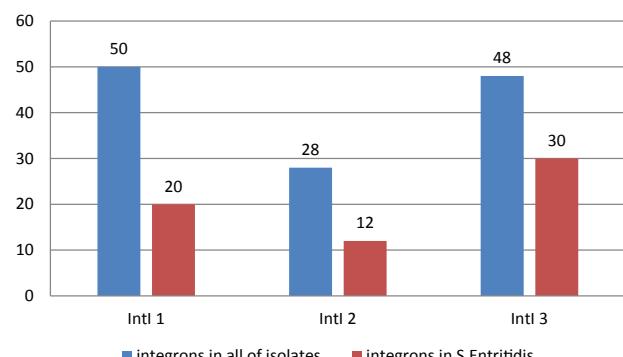
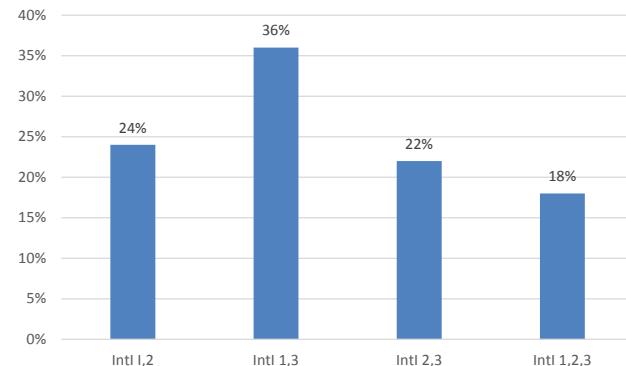


Figure 2. PCR results of Sef A primer. 1: marker (100 bp); 2: Negative control; 3-6: examined strains.



Graph 1. The prevalence of integrons 1 and 2, 3 in all of isolated and in *S. enteritidis*.



Graph 2. Percent of simultaneous occurrence of integron in all of isolates.

Table 2. Comparison of class1integrons effect on the resistance to different antibiotics.

Antimicrobial Agent	Antimicrobial resistance	R (%)	I (%)	S (%)	P value
Trimethoprim-sulfamethoxazole	IntI 1-positive	0	0	100	NS
	IntI 1-negative	0	0	100	
Ciprofloxacin	IntI 1-positive	0	0	100	NS
	IntI 1-negative	0	0	100	
Gentamicin	IntI 1-positive	16	84	0	**
	IntI 1-negative	2.6	11.2	86.2	
Nalidixic acid	IntI 1-positive	0	100	0	NS
	IntI 1-negative	0	100	0	
Chloramphenicol	IntI 1-positive	0	72	28	**
	IntI 1-negative	0	3.8	96.2	
Tetracycline	IntI 1-positive	16	0	84	*
	IntI 1-negative	2.5	0	97.5	
Clindamycin	IntI 1-positive	100	0	0	NS
	IntI 1-negative	100	0	0	
Ampicillin	IntI 1-positive	100	0	0	NS
	IntI 1-negative	100	0	0	
Cephalexin	IntI 1-positive	100	0	0	NS
	IntI 1-negative	100	0	0	

R _ Resistant; I _ Intermediate resistant; S _ susceptible; NS _ Resistance difference was not significant; ** _ Resistance difference was significant ($p<0.01$); * _ Resistance difference was significant ($p<0.05$)

According to Table 2, the antibiotic resistance to Gentamycin and Tetracycline in samples without IntI 1 were 2.6% and 2.5%, while examples of IntI1-positive have increased by 16% and the difference is significant ($p<0.01$). The strains intermediate resistant to Chloramphenicol in the samples that the IntI 1 were negative, were 3.8% while species intermediate resistant to Chloramphenicol were 72% in the samples IntI1-positive.

The resistance to Gentamicin were 32.4% from samples IntI 2-negative and this were 35.7% from strains positive IntI2. The resistance to Tetracycline was 21.4% in IntI2-positive while in IntI2-negative was 3.9% (Table 3).

Interestingly, 21.3% from strain that have IntI3 shown

Table 3. Comparison of class 2 integrons effect on the resistance to different antibiotics.

Antimicrobial Agent	Antimicrobial resistance	R (%)	I (%)	S (%)	P value
Trimethoprim-sulfamethoxazole	<i>IntI</i> 2-positive	0	0	100	NS
	<i>IntI</i> 2-negative	0	0	100	
Ciprofloxacin	<i>IntI</i> 2-positive	0	0	100	NS
	<i>IntI</i> 2-negative	0	0	100	
Gentamicin	<i>IntI</i> 2-positive	35.7	64.3	0	**
	<i>IntI</i> 2-negative	32.4	0	67.6	
Nalidixic acid	<i>IntI</i> 2-positive	0	100	0	NS
	<i>IntI</i> 2-negative	0	100	0	
Chloramphenicol	<i>IntI</i> 2-positive	0	42.9	57.1	NS
	<i>IntI</i> 2-negative	0	26.5	73.5	
Tetracycline	<i>IntI</i> 2-positive	21.4	0	78.6	**
	<i>IntI</i> 2-negative	3.9	0	96.1	
Clindamycin	<i>IntI</i> 2-positive	100	0	0	NS
	<i>IntI</i> 2-negative	100	0	0	
Ampicillin	<i>IntI</i> 2-positive	100	0	0	NS
	<i>IntI</i> 2-negative	100	0	0	
Cephalexin	<i>IntI</i> 2-positive	100	0	0	NS
	<i>IntI</i> 2-negative	100	0	0	

R_ Resistant; I_ Intermediate resistant; S_ susceptible; NS_ Resistance difference was not significant; **_ Resistance difference was significant ($p < 0.01$); * _ Resistance difference was significant ($p < 0.05$)

Table 4. Comparison of class3 integrons effect on the resistance to different antibiotics.

Antimicrobial Agent	Antimicrobial resistance	R (%)	I (%)	S (%)	P value
Trimethoprim-sulfamethoxazole	<i>IntI</i> 3-positive	0	0	100	NS
	<i>IntI</i> 3-negative	0	0	100	
Ciprofloxacin	<i>IntI</i> 3-positive	0	0	100	NS
	<i>IntI</i> 3-negative	0	0	100	
Gentamicin	<i>IntI</i> 3-positive	21.3	78.7	0	**
	<i>IntI</i> 3-negative	0	16.9	83.1	
Nalidixic acid	<i>IntI</i> 3-positive	0	100	0	NS
	<i>IntI</i> 3-negative	0	100	0	
Chloramphenicol	<i>IntI</i> 3-positive	0	57.4	42.6	**
	<i>IntI</i> 3-negative	0	14.5	85.5	
Tetracycline	<i>IntI</i> 3-positive	17	0	83	*
	<i>IntI</i> 3-negative	2.4	0	97.6	
Clindamycin	<i>IntI</i> 3-positive	100	0	0	NS
	<i>IntI</i> 3-negative	100	0	0	
Ampicillin	<i>IntI</i> 3-positive	100	0	0	NS
	<i>IntI</i> 3-negative	100	0	0	
Cephalexin	<i>IntI</i> 3-positive	100	0	0	NS
	<i>IntI</i> 3-negative	100	0	0	

R_ Resistant; I_ Intermediate resistant; S_ susceptible; NS_ Resistance difference was not significant; **_ Resistance difference was significant ($p < 0.01$); * _ Resistance difference was significant ($p < 0.05$)

antibiotic resistance to Gentamicin while none have shown the strains without integrons 3 resistance to these antibiotics. In addition resistance to Tetracycline has increased from 2.4% in strains *IntI*3-negative to 17% in *IntI*3-positive (Table 4).

4. Discussion

Multidrug-resistant bacteria pathogens such as *Salmonella* have become a major human health concern in developed and developing countries [17-19]. *Salmonella* has a broad vertebrate host range exists and most *Salmonella* disease are zoonotic [6,20]. The pollution of food products and the infection of foods of animal origin with *Salmonella* isolates carrying antibiotic resistance genes is an increasing concern [21]. *Salmonella* transmit by commercial industrial production of eggs, meat and food chains and can transfer resistance genes to human internal flora [1,6,20]. In recent years MDR among *Salmonella* is increasing, there for monitoring genotypic and phenotypic resistance to antibiotics in *Salmonella* is important for the public health [22,23]. In Iran, Japan and China have reported a high frequency of resistance to nalidixic acid and ciprofloxacin for *S. enterica* and *S. infantis* [24-27]. Asgharpour et al. [1] founded that all *Salmonella* isolates with MDR patterns had class 1 integron gene and showed that all of them were sensitive to Nalidixic acid, Streptomycin and Tetracycline [1]. In similar study, Kargar et al. [11] prevalent of class 1, 2 and 3 integrons in *Escherichia coli* were estimated as 78.26%, 76.81% and 26.09% [11]. The study in Armenia had shown the high presence of class 1 integrons and associated drug resistance among the *S. enterica* isolates [19]. According to other studies from Iran, the occurrence of infection and MDR to *Salmonella* is increasing in human and class 1 integrons more prevalent than class 2 in *Salmonella* isolates and to be associated with MDR [18,28]. The study in 2008, shown the high frequency of occurrence class 1 integrons in *Salmonella* strains and a strong association of with identified resistance antibiotics were demonstrated [29].

In this study similar to other reports, the prevalence of *intI1* (50%) was higher than the other two classes, in contrast to other studies, *intI3* (48%) is the second highest rate and *intI2* (28%) had the lowest prevalence among *Salmonella* isolated. The increasing presence *intI3* in *Salmonella* is concerned. Presence of integrons in *S. enteritidis* is more than total samplers. In this study, *intI2* associated with an integrons or two others. Based on the results of the antibiogram test, resistance to antibiotics direct associated with presence of integrons. Resistance to antibiotics Gentamicin, Tetracycline and Chloramphenicol associated with the presence of integrons. Increasing the presence of integrons in *Salmonella* and its relation to resistance to these antibiotics is concerned. Attention to the resistance of all samples of *Salmonella* isolates to antibiotics Clindamycin, Clindamycin and Cephalexin the use of antibiotics is not recommended in the treatment of *Salmonella*.

5. Conclusion

In conclusions, receipt and dissemination of antibiotic resistance genes in integrons has potential role in distribution of MDR. The results showed high prevalence class 1, 2 and 3 integrons and also high percentage of antibiotic resistance in *Salmonella*.

6. Authors' Contributions

AD conceived and designed the study. MDI carried out the laboratory work. MF participated in statistical analysis and procedures. NZJ coordinated and participated in designing the study. All the authors read and approved the final version.

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