Article

The evaluation of class 1 to 3 integrons in *Salmonella* and antimicrobial resistance pattern isolated from Ross 308 broiler chickens

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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*.

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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 (Asgharpour et al., 2014). 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) of *Salmonella* are considered as a threat for human's health (PAL et al., 2014). The genus of *Salmonella* belongs to the Enterobacteriaceae family and includes more than 2500 different serotypes, or serovars (Chu et al., 2008). These bacteria are rod-shaped, gram-negative, mesophilic, non-spore-

forming, predominantly motile and peritrichousflagella (PAL et al., 2014). *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 (Capalonga et al., 2014). 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 (Ranjbar et al., 2007). Antibiotic resistance of *Salmonella* is a major threat to human health and veterinary worldwide (Feasey et al., 2012; Forshell and Wierup, 2006). Currently, the indiscriminate use and misuse of antibiotics has facilitated the emergence of resistance in many *Salmonella* serovars (Angulo and Mølbak, 2005). Virulence factors and resistance may be present on chromosomes, plasmids, transposons, integronsor phage located and they are able to integrate and express genes coding for antibiotic resistance (Asgharpour et al., 2014).

Integrons are genetic elements that lead to various combinations of MDR pattern (Povilonis et al., 2010). Horizontal transfers of integrons are an important factor of MDR in gram-negative bacteria (Collis et al., 2002; Kargar et al., 2014). 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 (Gillings et al., 2008; Xu et al., 2007). Class 1 and 2 integrons were found to be the most common and are referred to as resistance integrons but class 3integrons are rare (Jin and Ling, 2009). Class 1 integrons are the most abundant type of integrons which are important for the creation and transfer of antibiotic resistance (Asgharpour et al., 2014). All integrons characterized are composed of three key elements necessary: a gene encoding an integrase belonging to the tyrosine-recombinase family (*intI*), a primary recombination site (*attI*) and promoter (Pc) (Mazel, 2006). 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 Material 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 (Asgharpour et al., 2014).

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 (2014). Each 50 µL reaction mixtures contained 10X reaction buffer, 0.5 µL dNTP mixture and 1.5 µL $MgCl_2$ along with 0.5 unit *Taq*DNA polymerase, 0.75 µL of each primer (class 1, 2 and 3 integrins) and 2.5 µL of template DNA. For negative control a tube containing PCR reaction without any DNA template was used (Kargar et al., 2014).

To identify genes related to the genera and species of enteritidis in *Salmonella Sef A* primers were used. Primer sequences and PCR conditions were shown in Table 1. PCR amplification was performed in a 25 µL reaction volume containing 1 µg of genomic DNA, 1 µM of each primer (forward and reverse of *Sef A*), 2 mM $MgCl_2$, 200 µM dNTP, 2.5 µL 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 (Doosti et al., 2014).

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 µg), Gentamicin (GM=10 µg), Ciprofloxacin (CP=5 µg), Nalidixic acid (NA=30 µg), Chloramphenicol (C=30 µg),

Tetracycline (tet=30 µg), Clindamycin (DA=2 µg), Ampicillin (AMP=10 µg), Cephalexin (CL=30 µg) (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**.**

Table 1 Primers and PCR conditions used in this study.

Primer name	Primer sequence	Observed size (bp)	PCR conditions
***IntI 1*-F**	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
***IntI 1*-R**	GGTCAAGGATCTGGATTTCG		
***IntI 2*-F**	CACGGATATGCGACAAAA AGG	788	
***IntI 2*-R**	ACATGCGTGTAAATCATC GTC		
***IntI 3*-F**	TGTTCTTGTATCGGCAGGTG	588	
***IntI 3*-R**	AGTGGGTGGCGAATGAGTG		
***Sef A*-F**	TGCTATTTTGCCCTGTACACTCG	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
***Sef A*-R**	TTCGGGGAGACTATACCTACAG		

3 Results

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

The PCR results in Fig. 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 integrons1, 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 (Fig. 3).

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% (Fig. 4).

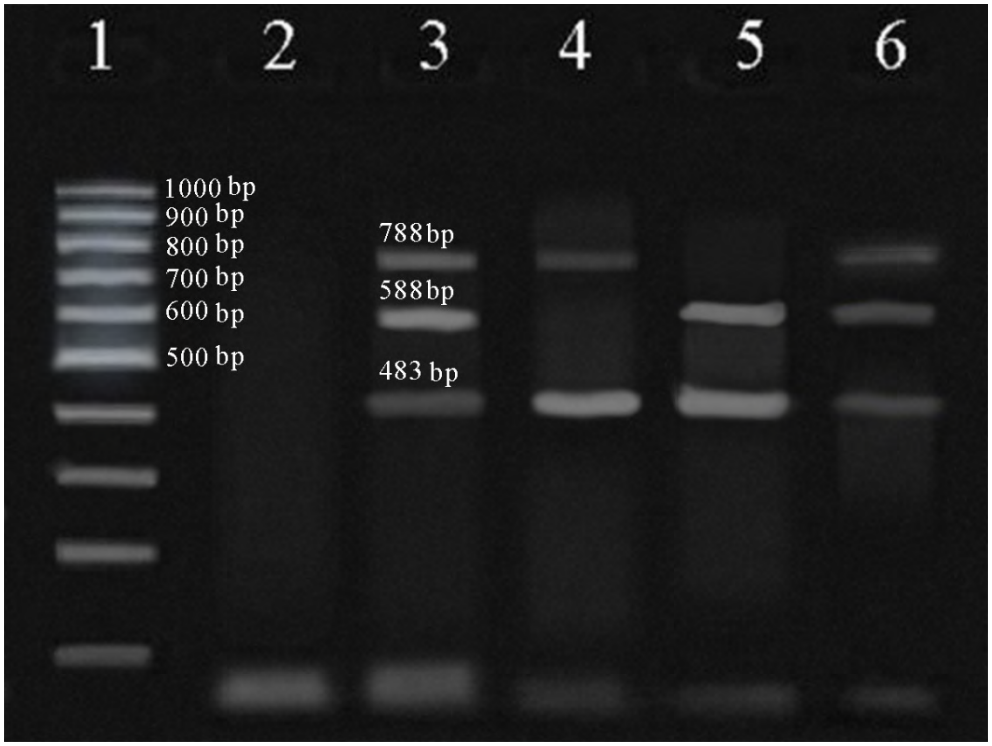

Fig. 1 Multiplex-PCR results of three integrons. 1: Marker (100 bp); 2: Negative control.

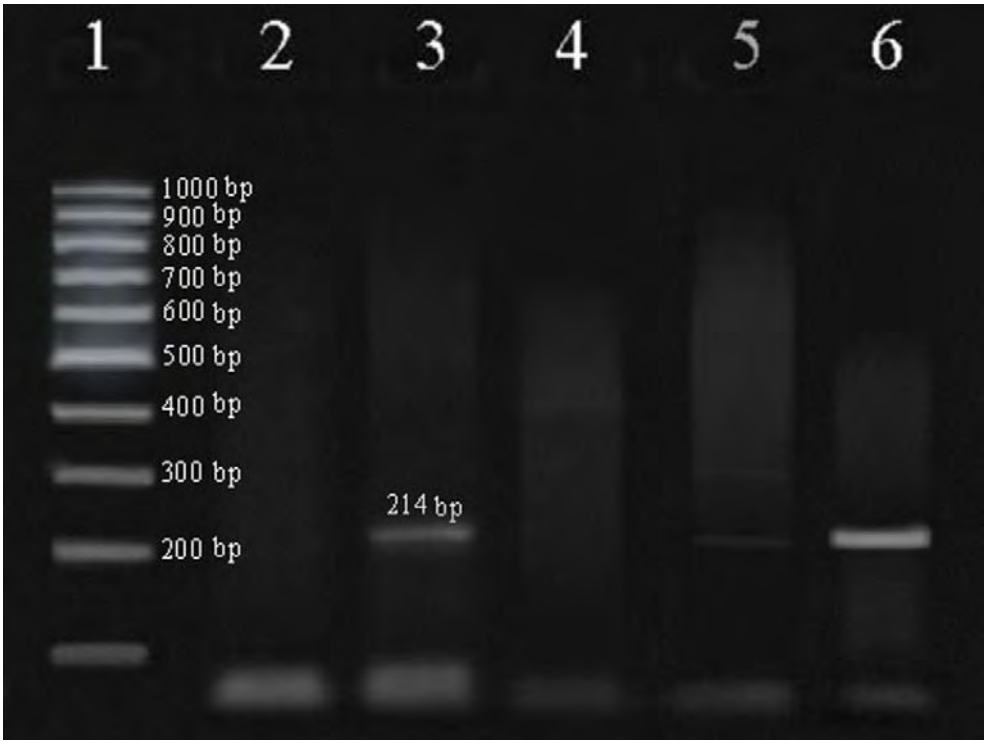

Fig. 2 PCR results of *Sef A* primer. 1: Marker (100 bp); 2: Negative control; 3-6: Examined strains.

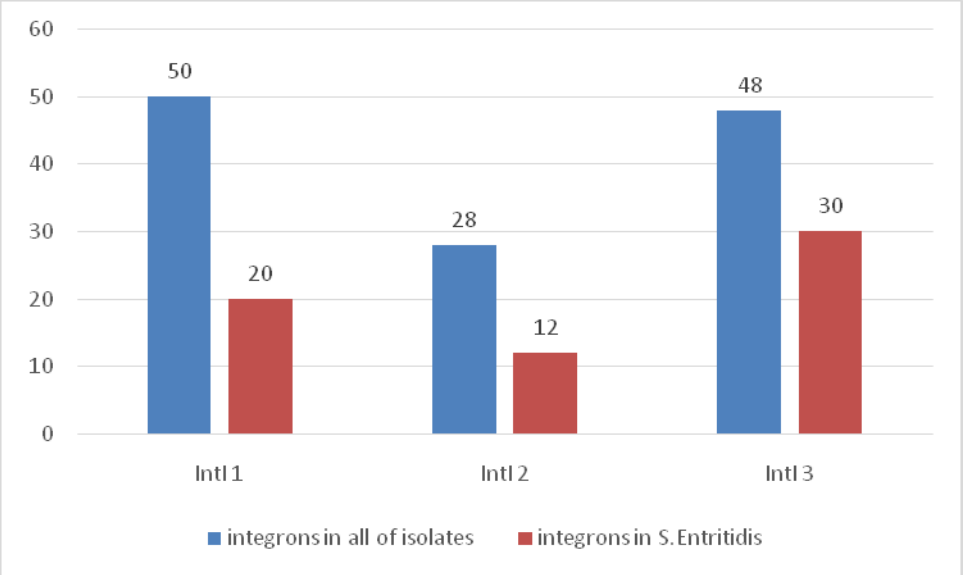

Fig. 3 The prevalence of integrons 1 and 2, 3 in all of isolated and in *S. entritidis*.

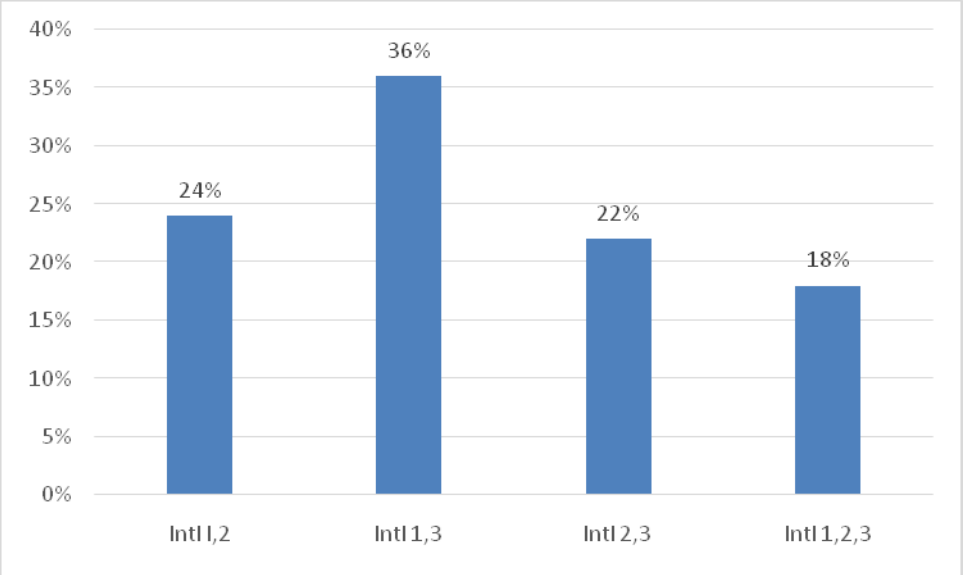

Fig. 4 Percent of simultaneous occurrence of integron in all of isolates.

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.

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 *IntI 1*-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 *IntI 1*-positive.

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

Antimicrobial Aent	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 ($p< 0.05$).

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

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

Antimicrobial Aent	Antimicrobial resistance	**R (%)**	**I (%)**	**S (%)**	**P value**
Trimethoprim-sulfamethoxazole	*IntI 2*-positive	0	0	100	NS
	IntI 2-negative	0	0	100	
Ciprofloxacin	*IntI 2*-positive	0	0	100	NS
	IntI 2-negative	0	0	100	
Gentamicin	*IntI 2*-positive	35.7	64.3	0	**
	IntI 2-negative	32.4	0	67.6	
Nalidixic acid	*IntI 2*-positive	0	100	0	NS
	IntI 2-negative	0	100	0	
Chloramphenicol	*IntI 2*-positive	0	42.9	57.1	NS
	IntI 2-negative	0	26.5	73.5	
Tetracycline	*IntI 2*-positive	21.4	0	78.6	**
	IntI 2-negative	3.9	0	96.1	
Clindamycin	*IntI 2*-positive	100	0	0	NS
	IntI 2-negative	100	0	0	
Ampicillin	*IntI 2*-positive	100	0	0	NS
	IntI 2-negative	100	0	0	
Cephalexin	*IntI 2*-positive	100	0	0	NS
	IntI 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 ($p< 0.05$).

Interestingly, 21.3 % from strain that have *IntI 3* shown 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).

Table 4 Comparison of class 3 integrons effect on the resistance to different antibiotics.

Antimicrobial Aent	Antimicrobial resistance	**R (%)**	**I (%)**	**S (%)**	**P value**
Trimethoprim-sulfamethoxazole	*IntI 3*-positive	0	0	100	NS
	IntI 3-negative	0	0	100	
Ciprofloxacin	*IntI 3*-positive	0	0	100	NS
	IntI 3-negative	0	0	100	
Gentamicin	*IntI 3*-positive	21.3	78.7	0	**
	IntI 3-negative	0	16.9	83.1	
Nalidixic acid	*IntI 3*-positive	0	100	0	NS
	IntI 3-negative	0	100	0	
Chloramphenicol	*IntI 3*-positive	0	57.4	42.6	**
	IntI 3-negative	0	14.5	85.5	
Tetracycline	*IntI 3*-positive	17	0	83	*
	IntI 3-negative	2.4	0	97.6	
Clindamycin	*IntI 3*-positive	100	0	0	NS
	IntI 3-negative	100	0	0	
Ampicillin	*IntI 3*-positive	100	0	0	NS
	IntI 3-negative	100	0	0	
Cephalexin	*IntI 3*-positive	100	0	0	NS
	IntI 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 ($p < 0.05$).

4 Discussion

Multidrug-resistant bacteria pathogens such as *Salmonella* have become a major human health concern in developed and developing countries (Chen et al., 2004; Naghoni et al., 2010; Zakharyan et al., 2014). *Salmonella* has a broad vertebrate host range exists and most *Salmonella* disease are zoonotic (Anjum et al., 2011; Feasey et al., 2012). The pollution of food products and the infection of foods of animal origin with *Salmonella* isolates carrying antibiotic resistance genes is an increasing concern (Lopes et al., 2014). *Salmonella* transmit by commercial industrial production of eggs, meat, and food chains and can transfer resistance genes to human internal flora (Anjum et al., 2011; Asgharpour et al., 2014; Feasey et al., 2012). 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 (Hoelzer et al., 2011; Ribeiro et al., 2011). In Iran, Japan and China have reported a high frequency of resistance to nalidixic acid and ciprofloxacin for *S. enterica* and *S. infantis* (Dahshan et al., 2010; Morshed and Peighambari, 2010; Rahmani et al., 2013; Yan et

al., 2010). Asgharpour et al. (2014) found that all *Salmonella* isolates with MDR patterns had class 1integron gene and showed that all of them were sensitive to Nalidixic acid, Streptomycin and Tetracycline (Asgharpour et al., 2014). In similar study, Kargar et al. (2014) found that the prevalence of class 1, 2 and 3 integrons in *Escherichia coli* were estimated as 78.26%, 76.81%, and 26.09% (Kargar et al., 2014). The study in Armenia had shown the high presence of class I integrons and associated drug resistance among the *S. enteric* isolates (Zakharyan et al., 2014). 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 (Bozorgmehri Fard et al., 2016; Naghoni et al., 2010). 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 (Rao et al., 2008).

In this study similar to other reports, the prevalence of *intI 1* (50%) was higher than the other two classes, in contrast to other studies, *intI 3* (48%) is the second highest rate and *intI 2* (28%) had the lowest prevalence among *Salmonella* isolated. The increasing presence *intI 3* in *Salmonella* is concerned. Presence of integrons in *S. enteritidis* is more than total samplers. In this study, *intI 2* 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*.

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