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Antibiotic Susceptibility and Molecular Screening of Class I Integron in *Salmonella* Isolates Recovered from Retail Raw Chicken Carcasses in China

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Salmonella is one of the leading causes for foodborne diseases. Foods, particularly those of animal origin, act as an important role for *Salmonella* transmission. In this study, the antibiotic susceptibility of 743 *Salmonella* isolates recovered from retail raw chicken carcasses in eight provinces was tested, and the isolates were also screened for the presence of class I integron and drug-resistant gene cassettes. One hundred thirteen (15.21%) isolates were harboring class I integron. A higher percentage of integron-positive *Salmonella* isolates were found in retail chicken in Sichuan Province (29.33%), followed by Beijing (22.14%), Shaanxi (19.15%), Guangxi (14.13%), Henan (12.50%), Shanghai (7.25%), Fujian (8.22%), and Guangdong (6.25%) Provinces. The respective prevalence of class I integron in *Salmonella* isolates recovered from retail chickens in large, free, and small markets was 16.31%, 14.04%, and 15.27%. Moreover, 20.13%, 14.02%, and 13.74% of *Salmonella* isolates recovered from retail chickens stored in frozen, chilled, and ambient conditions, respectively, were positive for class I integron. Subsequent sequencing of class I integron revealed the presence of 10 gene cassettes harboring resistance genes (*dfr*A17-*aad*A5, *dfr*A17-*aad*A5, *dfr*A1-*aad*A1, *dfr*A12-*aad*A2, *dfr*A17-*aad*A5-*aad*A4, *dfr*A1-*aad*A1-*aad*A2, *dfr*A1, *dfr*A5, *aad*A2, *aac*A4-*cat*B8-*aad*A1-*dfr*A1-(*aac*6-II)-(bla_{CARB}-8), bla_{PSE-1}-bla_{P1}). The most prevalent gene cassette was *dfr*A17-*aad*A5 (59.62%). Class I integron-positive isolates were significantly more resistant to multiple antibiotics, and they commonly exhibited corresponding antibiotic resistance profiles to the antibiotic resistance gene cassettes harbored in their class I integron. The results indicated that class I integron with different antibiotic resistance gene cassettes that were prevalent in *Salmonella* isolates differed from provinces, marketplaces, and chicken storage conditions.

Introduction

Salmonella is a notorious pathogen associated with foodborne outbreaks worldwide.[1] *Salmonella* infections were reported as the second leading cause of bacterial foodborne illness in the United States[2] and were responsible for ~1.2 million patients annually, resulting in estimated medical costs of $365 million.[3] In the European Union, salmonellosis was the second most commonly reported gastrointestinal infection, with a confirmed case rate of 20.4 cases per 100,000 individuals in 2011.[4] In China, 22.2% of foodborne diseases were caused by *Salmonella* spp.,[5] and salmonellosis was ranked as the fourth most prevalent foodborne disease caused by microbial agents.[6]

Among various foods, poultry and poultry products (meat and eggs) had been recognized as the most significant vehicle for human salmonellosis,[7] and several foodborne *Salmonella* outbreaks were caused by contaminated poultry products.[8,9] Antibiotics would remain the most effective treatment for invasive, life-threatening salmonellosis infection for a long time in the future.[10] However, ubiquitous usage of antibiotics not only facilitated the emergence of antibiotic resistance but also created significant challenges to animal husbandry.[10,11] Several previous foodborne outbreaks indicated that the multidrug-resistant (MDR) *Salmonella* was of increasing concern.[12–15]

Integrons are mobile DNA elements comprising a site-specific recombination system that is capable of integrating and expressing antibiotic resistance genes in cassette-like structures and transferring resistant genes from one bacterium to another, which in turn lead to the emergence of antibiotic resistance.[15,16] Among various integrons, class I integron

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was the most predominant one and played a major role in multidrug resistance development of *Salmonella*.[17–20]

Several previous studies indicated that class I integrons could be detected in MDR *Salmonella* from animals, eggs, poultry, human, pork, retail beef, and chicken carcasses in Japan, the United States, Iran, Ethiopia, Taiwan, Brazil, and China.[8,12,16,17,19–22] However, information regarding the distribution of antimicrobial resistance-associated integron in *Salmonella* among retail foods is still not well documented. In this study, the prevalence of class I integron and antibiotic resistance-associated gene cassettes in *Salmonella* isolates were determined for better understanding class I integron distribution in *Salmonella* isolates recovered from retail chicken carcasses in China.

Materials and Methods

Salmonella *isolates*

Seven hundred forty-three *Salmonella* isolates (73 isolates were from Fujian, 75 from Sichuan, 88 from Henan, 92 from Guangxi, 94 from Shaanxi, 112 from Guangdong Province, 69 from Shanghai, and 140 from Beijing) were included in this study. These isolates were recovered from retail raw chicken carcasses collected from different marketplaces in six provinces and two national cities in China during 2010 and 2011. Detailed information on sample collection, *Salmonella* isolation, and identification were as previously described.[23,24]

Antimicrobial susceptibility test

All *Salmonella* isolates were tested for their susceptibility to antimicrobials. The minimum inhibitory concentrations (MICs) of the antimicrobial agents were determined by the agar dilution method using Mueller–Hinton agar (Beijing Land Bridge Technology Co Ltd., Beijing, China) according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI).[25] The antibiotics include ampicillin (AMP), amoxicillin–clavulanic acid (AMC), ceftiofur (TIO), ceftriaxone (CRO), cefoxitin (FOX), gentamicin (GEN), kanamycin (KAN), amikacin (AMK), streptomycin (STR), tetracyclines (TCY), sulfisoxazole (FIS), trimethoprim–sulfamethoxazole (SXT), chloramphenicol (CHL), nalidixic acid (NAL), and ciprofloxacin (CIP). *Escherichia coli* ATCC25922 and ATCC35218, *Enterococcus faecalis* ATCC29212 were used as quality control organisms in MIC determination. The breakpoints for the antibiotics were as the interpretive standards provided by CLSI,[25] except for streptomycin, for which we used provisional breakpoint used by the National Antimicrobial Resistance Monitoring System (NARMS).[26]

Molecular detection of class I integron

All *Salmonella* isolates were screened for the presence of class I integron by PCR using the primers of intI-F-(5′CS)-5′-GGCATCCAAGCAGCAAG-3′ and intI-R-(3′CS)-5′-AAGCAGACTTGACCTGA-3′.[27,28] The PCRs were carried out in a Mycircle PCR system (Bio-Rad, Hercules, CA) in a 25 μl PCR mixture that contained 0.5 μM of each primer, 1×PCR buffer, 250 μM of dNTP, 0.5 U of Taq DNA polymerase (TaKaRa, Dalian, China), 1.5 mM $MgCl_2$, and 5 μl of sample template DNA. The PCR conditions were at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, and a final extension of 72°C for 10 min.

DNA sequencing and resistance gene cassette analysis

PCR products were purified using the TaKaRa Agarose Gel DNA Purification Kit (Version 2.0; TaKaRa), then stored in an ice box, and sent for sequencing at Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China). DNA sequence data were analyzed and aligned using BLAST program (http://ncbi.nlm.nih.gov/BLAST/).

Statistical analysis

The prevalence data of class I integron in *Salmonella* isolates were subjected to DPS software (version 9.5, Institute of Insect Science, Zhejiang University, Hangzhou, People's Republic of China) to determine the significant variation. Significant ($p<0.05$) difference of the prevalence of class I integron in *Salmonella* isolates in different provinces and cities, different retail chicken storage conditions, and different marketplaces was determined using the Pearson's chi-squared test at the 5% level ($\alpha=0.05$).

Results

Among 743 *Salmonella* isolates, a higher percentage of the isolates (77.12%) were resistant to tetracycline, followed by sulfisoxazole (66.62%), nalidixic acid (64.74%), ampicillin (61.37%), trimethoprim–sulfamethoxazole (56.53%), and amoxicillin–clavulanic acid (48.45%) (Table 1). One hundred thirteen (15.2%) of the total isolates were harboring class I integron of 2 kb. *Salmonella* isolates harboring class I integron presented a significantly ($p<0.05$) higher resistance to tetracycline, ampicillin, trimethoprim–sulfamethoxazole, amoxicillin–clavulanic acid, chloramphenicol, kanamycin, gentamicin, ceftiofur, cefoxitin, and amikacin compared with the average resistance rates (Table 1).

Table 2 clarified that in all 113 class I integron-positive isolates, 5 (4.42%), 23 (20.35%), 37 (32.74%), 37 (32.74%), and 8 (7.08%) isolates showed resistance to 0, 2–5, 6–9, 10–13, and 14–17 tested antibiotics, respectively. Class I integron-positive isolates presented significantly ($p<0.05$) more resistance to multiple antibiotics compared with the average rates of *Salmonella*.

The results clarified a higher prevalence rate (29.33%) of class I integron-positive *Salmonella* isolates recovered from retail chicken carcasses in Sichuan Province and this rate was significantly higher ($p<0.05$) than those in Guangxi (14.13%), Henan (12.50%), Fujian (8.22%), Shanghai (7.25%), and Guangdong (6.25%) Provinces. Moreover, there was no significant difference ($p>0.05$) in the detection rates of class I integron-positive isolates in Sichuan (29.33%), Beijing (22.14%), and Shaanxi (19.15%) Provinces. Collectively, our results showed a significantly ($p<0.05$) higher prevalence rate (20.65%) of class I integron-positive *Salmonella* isolates in the Northern Provinces of China (Henan, Shaanxi, Sichuan, and Beijing) compared to (8.96%) the Southern ones (Fujian, Guangdong, Guangxi, and Shanghai).

No significant difference was found among the prevalence rates of Class I integron in *Salmonella* isolates recovered

Table 1. Antimicrobial Resistance of the *Salmonella* Isolates

Antimicrobial agent	*No. (%) of antimicrobial-resistant isolates (n = 743)*	*No. (%) of antimicrobial-resistant isolates carrying class I integron (n = 113)*
Aminoglycosides		
Amikacin	138 (18.57) A	44 (38.94) B
Gentamicin	269 (36.20) A	60 (53.10) B
Kanamycin	309 (41.59) A	63 (55.75) B
Streptomycin	223 (30.01) A	29 (25.66) A
Cephalosporins		
Ceftiofur	193 (25.98) A	53 (46.90) B
Cefoxitin	141 (18.98) A	43 (38.05) B
Ceftriaxone	126 (16.96) A	22 (19.47) A
Penicillin β-lactamase inhibitor combinations		
Ampicillin	456 (61.37) A	85 (75.22) B
Amoxicillin–clavulanic acid	360 (48.45) A	78 (69.03) B
Quinolone		
Nalidixic acid	481 (64.74) A	56 (49.56) B
Fluoroquinolones		
Ciprofloxacin	170 (22.88) A	16 (14.16) B
Gatifloxacin	129 (17.36) A	15 (13.27) A
Levofloxacin	135 (18.17) A	16 (14.16) A
Folate pathway inhibitors		
Sulfisoxazole	495 (66.62) A	78 (69.03) A
Trimethoprim–sulfamethoxazole	420 (56.53) A	83 (73.45) B
Chloramphenicol		
Chloramphenicol	327 (44.01) A	76 (67.26) B
Tetracycline		
Tetracycline	573 (77.12) A	100 (88.50) B

Ratios followed by different letters were significantly different ($p < 0.05$).

from retail chickens stored at frozen (20.13%), chilled (14.02%), and ambient (13.74%) conditions. In addition, class I integron was more prevalent in *Salmonella* strains isolated from chickens stored at frozen conditions in Beijing (10.39%) and less prevalent in Henan Province (0.65%). On the contrary, class I integron was found in *Salmonella* recovered from chicken carcasses stored at chilled conditions in the Sichuan Province (2.38%) and 3.79% in chickens stored at ambient temperature in the Beijing Province.

Regarding the distribution of class I integron in *Salmonella* spp. isolated from chickens at different marketplaces, Table 3 clarified that 16.31%, 15.27%, and 14.04% of the total isolates recovered from chickens at large, free, and small markets, respectively, were harboring class I integron. In addition, in *Salmonella* strains isolated from chickens of large markets, class I integron was more prevalent in Shaanxi Province (3.86%) than in other Provinces. Meanwhile, for small markets and free markets, the highest prevalence rates of class I integron were both detected in Beijing, with a ratio of 5.96% and 4.03%, respectively. Moreover, there was no significant difference ($p > 0.05$) in the detection rates of class I integron-positive isolates between capital cities and noncapital cities (Table 3).

Among the 113 class I integron-positive *Salmonella* isolates, nine (7.96%) were carrying no antibiotic-resistant gene cassettes (Table 4). Approximately 8.85% of class I integron carried one antibiotic resistance gene, and 83.19% of

Table 2. Multidrug Resistance of *Salmonella* Isolates Against 17 Antimicrobials

Number of antimicrobials	*No. (%) of isolates resistant to indicated number of antimicrobials (n = 743)*	*No. (%) of class I integron-positive isolates resistant to indicated number of antimicrobials (n = 113)*
0	38 (5.11) A	5 (4.42) A
1	43 (5.79) A	3 (2.65) A
2–5	284 (38.22) A	23 (20.35) B
6–9	177 (23.82) A	37 (32.74) B
10–13	111 (14.94) A	37 (32.74) B
14–17	90 (12.11) A	8 (7.08) B

Ratios followed by different letters were significantly different ($p < 0.05$).

Table 3. Distribution of Class I Integron in *Salmonella* Isolates Recovered from Retail Chickens Collected in Different Districts, Marketplaces, and Storage Conditions

	No. isolates	*No. class I integron-positive isolates*	*% class I integron-positive isolates*
Province/national city			
Sichuan	75	22	29.33 A
Beijing	140	31	22.14 AB
Shaanxi	94	18	19.15 AB
Guangxi	92	13	14.13 BC
Henan	88	11	12.50 BC
Fujian	73	6	8.22 C
Shanghai	69	5	7.25 C
Guangdong	112	7	6.25 C
Chicken storage condition			
Frozen	154	31	20.13 A
Chilled	378	53	14.02 A
Ambient	211	29	13.74 A
Marketplace			
Large market	233	38	16.31 A
Small market	235	33	14.04 A
Free market	275	42	15.27 A
Capital city/noncapital city			
Capital city	161	24	14.91 A
Noncapital city	582	89	15.29 A
Northern/Southern province (city) of China[a]			
Southern province (city)	346	31	8.96 B
Northern province (city)	397	82	20.65 A

Ratios followed by different letters were significantly different ($p < 0.05$).

[a]Northern provinces (city), including Shaanxi, Sichuan, Henan Provinces, and Beijing city; Southern provinces (city), including Guangdong, Guangxi, Fujian Provinces, and Shanghai city.

Table 4. Presence of Antibiotic Resistance Gene Cassettes in Class I Integron-Positive *Salmonella* in Retail Chicken Carcasses ($N=113$)

Antibiotic resistance gene cassettes	*No. class I integron-positive isolates*	*% class I integron- positive isolates*
*dfr*A17-*aad*A5	62	54.87
*dfr*A1-*aad*A1	24	21.24
*dfr*A1	7	6.19
*dfr*A12-*aad*A2	3	2.65
*aad*A2	2	1.77
bla_{PSE}-1-bla_{P1}	2	1.77
*dfr*A5	1	0.88
*aac*A4-*cat*B8-*aad*A1-*dfr*A1-(*aac*6-II)-(bla_{CARB-8})	1	0.88
*dfr*A1-*aad*A1-*aad*A2	1	0.88
*dfr*A17-*aad*A5-*aad*A4	1	0.88
	9	7.96

class I integron simultaneously carried two or more resistance genes. In addition, 10 different gene cassettes containing 14 resistance genes were identified in 104 (92.04%) of *Salmonella* isolates. The cassettes included *dfr*A17-*aad*A5, *dfr*A1-*aad*A1, bla_{PSE-1}-bla_{P1}, *dfr*A1, *aad*A2, *dfr*A5, *dfr*A12-*aad*A2, *aac*A4-*cat*B8-*aad*A1-*dfr*A1-(*aac*6-II)-(bla_{CARB-8}), *dfr*A1-*aad*A1-*aad*A2, and *dfr*A17-*aad*A5-*aad*A4, respectively (Table 4). The most prevalent resistance gene cassette was *dfr*A17-*aad*A5 (54.87%), followed by *dfr*A1-*aad*A1 (21.24%) and *dfr*A1 (6.19%).

The results clarified that among class I integron-positive *Salmonella* isolates with *dfr* genes (*dfr*A1, *dfr*A5, *dfr*A12, and *dfr*A17), the predominant antimicrobial resistance profile was FIS-SXT (65.22%). Meanwhile, 31.40% of isolates harboring *aad* genes (*aad*A1, *aad*A2, *aad*A5, and *aad*A4) presented the profile of GEN-KAN-AMK. Among 11 class I integron-positive *Salmonella* isolates presenting antibiotic resistance profile of GEN-KAN-AMK-STR, at least one of the *aad* genes could be detected. Meanwhile, two class I integron-positive *Salmonella* isolates carrying bla_{PSE-1}-bla_{P1} genes were resistant to ceftiofur and ceftriaxone.

Discussion

Salmonella is a major cause of foodborne illness worldwide. The increasing frequency of antibiotic resistance among *Salmonella* has become an emerging challenge to public health.[29] In the present study, 89.10% of *Salmonella* isolates were resistant to two or more antimicrobial agents. These results were similar to those of other investigators.[30–32] Our study indicated that *Salmonella* isolates were most frequently resistant to tetracycline (77.12%), sulfisoxazole (66.62%), and nalidixic acid (64.74%), and this was consistent with the findings of other studies.[33,34] This study presented that 92.92% of the class I integron-positive isolates were resistant to at least two antibiotics, and this exhibited a good evidence for the relationship between the presence of class I integron and the emerging of MDR in gram-negative bacteria.[12,29]

Integron, a mobile DNA element, is known to contain one or more linked antimicrobial resistance genes and can transfer these genes among bacteria.[35,36] In this study, PCR analysis showed that 113 (15.21%) of 743 *Salmonella* isolates were carrying class I integron. In one of our previous studies, it was found that 16.0% of the *Salmonella* strains isolated from retail foods carried class I integron in Shaanxi province during the period 2007–2008.[37] Other previous surveys in China identified class I integron in *Salmonella* isolates recovered from retail foods, human, and animal sources with percentages that ranged from 11.0% to 66.0%.[21,22,38,39] Compared with these surveys, we were the first to investigate class I integron in *Salmonella* isolates in retail raw chickens by such large sample size and basis.

In the present study, there was a variation in the prevalence of class I integron in *Salmonella* isolates recovered from retail chickens of different provinces, national cities, storage conditions, and marketplaces. This variation could be attributed to the difference in the growth and slaughter environment of chickens.[38] In addition, medical qualities had certain varieties, which led to the difference of class I integron and resistance genes.[40] Chicken carcasses were often stored in the markets as fresh at ambient, or as packaged chilled products, or in frozen conditions. The difference in temperature, in which chickens were stored, might have influenced the number and types of *Salmonella,* and consequently affected the popularity of class I integron. In our study, class I integron was most frequently prevalent in *Salmonella* recovered from chicken carcasses that were stored in frozen conditions (20.13%) compared to 13.74% in chickens stored at ambient temperature. The previous studies had shown that *Salmonella* contamination in the raw chickens was related to temperature,[41] and our results indicated that the survived *Salmonella* isolates in frozen chicken carcass were more resistant and likely to be class I integron positive.

The former studies have indicated the existence of certain relationship between integrons and multidrug resistance of *Salmonella* strains, and provided certain significant guidelines for drug resistance prevention and control.[20,29] However, it was interesting that no resistance gene was detected in the variable region of class I integron in the two MDR isolates in this study. As it was known, antibiotic resistance associated with many factors, including antimicrobial agents binding target mutations, increased permeability of membrane of the pathogens, and antibiotic degradation through enzymes encoded by some resistant genes that were commonly carried by some transferable DNA elements, including conjugative plasmids, integrons, transposons, and phages.[9,18,21,35,42] Since any transferable element other than class I integron could play an important role for antimicrobial resistance gene carriage and dissemination as well, the resistance of the two isolates might be caused by other mechanisms instead of class I integron.[16,27,30]

Our results indicated that the isolates harboring class I integron were more resistant to the antibiotics. The *dfr*, *aad*, and *bla* genes have always contributed to the resistance to sulfonamide, aminoglycoside, and cephalosporin antibiotics, respectively. In this study, 91.3% of the 92 isolates carrying *dfr* genes (*dfr*A1, *dfr*A5, *dfr*A12, and *dfr*A17) showed resistance to sulfonamides. The resistance to aminoglycoside antibiotics was identified in 51 of the 86 isolates carrying *aad* genes (*aad*A1, *aad*A2, *aad*A5, and *aad*A4). In addition, two of the class I integron-positive isolates carrying bla_{PSE-1}-bla_{P1} genes were resistant to ceftiofur and ceftriaxone. Various

antibiotic resistance gene cassettes, including *dfr*A17-*aad*A5, *dfr*A1-*aad*A1, bla_{PSE-1}-bla_{P1}, *dfr*A1, *aad*A2, *dfr*A5, *dfr*A12-*aad*A2, *aac*A4-*cat*B8-*aad*A1-*dfr*A1-(*aac*6-II)-(*bla*CARB-8), *dfr*A1-*aad*A1-*aad*A2, and *dfr*A17-*aad*A5-*aad*A4, were found in integron, and *Salmonella* isolates with these gene cassettes in their class I integron generally exhibited corresponding antibiotic resistance profiles. The results inferred that the gene cassettes in the class I integron might be the major cause of antibiotic resistance in those *Salmonella*. In class I integron, antibiotic resistance gene cassettes were normally found integrated at a specific location, and enzymes encoded by the integron could make the gene cassettes transfer from one bacteria to another, or from one strain to other strains.[42–44] The presence of class I integron in *Salmonella* isolates is of great potential hazard to the health and food safety of humans and animals.

In summary, class I integron was ubiquitous in *Salmonella* isolates recovered from retail raw chicken carcasses and varied in different marketplaces and chicken storage conditions at different districts of China. *Salmonella* isolates harboring class I integron showed more resistance to the antibiotics than those without it. Two or more antibiotic resistance gene cassettes were prevalent in class I integron.

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Disclosure Statement

No competing financial interests exist.

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