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# Distribution and Characterization of Integrons in Enterobacteriaceae Isolates from Chickens in Korea

**Ji Youn Sung and Ji-Eun Oh***

*Department of Biomedical Laboratory Science, Far East University, Chungbuk-do 369-700, Republic of Korea*

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**Corresponding author*
Phone: +82-43-879-3668;
Fax: +82-43-880-3876;
E-mail: ohjieun8078@gmail.com

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The use of antimicrobial agents for additives or therapeutics is strongly associated with a prevalence of antimicrobial resistance in commensal Enterobacteriaceae. We aimed to characterize integrons in Enterobacteriaceae isolates obtained from chicken cecums in Korea. Moreover, the correlation between integron gene cassettes and antimicrobial resistance was also investigated. A total of 90 isolates the belonged to Enterobacteriaceae were recovered from chickens grown at Gyeongsang and Chungcheong provinces in Korea. Antimicrobial susceptibility tests were performed by the disk diffusion method. PCR and DNA sequencing were also performed to characterize the gene cassette arrays of the integrons. Of the 90 Enterobacteriaceae isolates tested, 39 (43.3%) and 10 (11.1%) isolates carried class 1 and 2 integrons, respectively. Whereas the class 2 integron did not contain gene cassettes, the class 1 integrons carried seven different gene cassette arrays. The class 1 integrons harbored genes encoding resistant determinants to aminoglycosides (*aadA1*, *aadA2*, and *aadA5*), trimethoprim (*dfrA1*, *dfrA12*, *dfrA17*, and *dfrA32*), lincosamides (*linF*), and erythromycin (*ereA*). Moreover, the presence of a class 1 integron was significantly related to a high resistance rate of antimicrobial agents, such as spectinomycin and trimethoprim. We confirmed that diverse class 1 integrons were widely distributed in Enterobacteriaceae isolates from chickens and directly contributed to the resistance to diverse antimicrobial agents in Korea.

**Keywords:** Enterobacteriaceae, integron, chicken, gene cassette, antimicrobial agents, resistance

## Introduction

Antimicrobial agents are extensively used to treat or prevent bacterial diseases in humans and animals, but the emergence of multidrug-resistant (MDR) bacteria has become a major public health concern worldwide. In particular, antimicrobial agents are added in the feed of healthy animals for several purposes, resulting in an increase of antimicrobial-resistant intestinal bacteria that may be transferred to humans by food chains. In this case, transferred animal intestinal bacteria could also disseminate antimicrobial-resistant genes to human intestinal bacterial flora through mobile elements like transposons and integrons [16, 10].

In 2011, the use of antimicrobial agents for the purpose of animal growth promoters was banned in Korea [11]. However, antimicrobial agents can still be added to water, food, and injected into animals with a veterinary prescription on individual farms [13].

The genera belonging to Enterobacteriaceae inhabit intestinal tracts of humans and animals, and are common pathogens causing serious nosocomial and community acquired infections in various organs and tissues. Moreover, it has been demonstrated that *Escherichia coli* and *Salmonella* spp. show high incidences of resistance of commercially available antimicrobial agents [8]. Therefore, these facts imply high risks of disseminating antimicrobial-resistant genes between bacterial species and, ultimately, to human.

Integrons are one of the important genetic platforms for gene cassettes encoding determinants of antimicrobial resistance. They frequently facilitate site-specific acquisitions and transmission of antimicrobial resistance determinants between inter- and intraspecies [7]. Five classes of intergrons

have been identified to date, and class 1 and 2 integrons are frequently found in bacteria. Class 1 integrons, the most common type, contain a 5′ conserved segment (5′CS) and a 3′ conserved segment (3′CS). The 5′CS consists of an integrase gene (*intI*), a recombination site (*attI1*), and a promoter region expressing the inserted gene(s). The 3′CS includes a defective quaternary ammonium resistance gene (*qacEΔ1*) and a sulfonamides resistance gene (*sul1*) [14, 15]. The variable region located in between 5′CS and 3′CS segments harbors various antimicrobial resistance gene cassettes encoding dihydroflavonol-4-reductase (*dfr*), broad-spectrum β-lactamase, and aminoglycoside-modifying enzymes such as acetyltransferase (*aac*), adenylyltransferase (*aad*), and phosphotransferase (*aph*) [2, 6]. Class 2 integrons, usually identified in Enterobacteriaceae, have an integrase gene (*intII*) and a recombination site, but no *sul1* gene in the 3′CS.

Although genetic mobile elements like integrons frequently transfer antimicrobial resistance determinants in gram-negative bacteria, only a few studies have investigated the frequencies and integron gene casssettes in animal bacteria from Korea. The aims of this study were to characterize the integrons responsible for antimicrobial resistance of Enterobacteriaceae isolates from chickens. In addition, the relationship between the gene cassette arrays of integron and antimicrobial resistance was analyzed.

## Materials and Methods

### Bacterial Isolates

A total of 90 isolates belonging to Enterobacteriaceae were recovered from cecums of chickens that were grown in Chungcheong and Gyeongsang provinces from July to December in 2013. The chicken cecums were obtained from their respective slaughterhouses.

Species identification was performed using the Vitek 2 automated instrument ID system (bioMérieux, Marcy l'Etoile, France), conventional methods, and analyses of their 16S rRNA sequences.

### Antimicrobial Susceptibility Test

All Enterobacteriaceae isolates were subjected to a susceptibility test against 12 antimicrobial agents on Müller-Hinton agar (Difco Laboratories, Detroit, MI, USA) with the Kirby-Bauer disk diffusion method. The following antimicrobial disks (BBL, Cockeysville, MI., USA) were used: amikacin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), spectinomycin (100 μg), streptomycin (10 μg), and trimethoprim (5 μg). According to the CLSI M100-S21 guidelines, inhibition zones of all antimicrobial disks were measured and evaluated as susceptible, intermediate, or resistant, except for the spectinomycin disk [1]. *E. coli* strain ATCC 25922 was used as a reference strain. The inhibition zone of the spectinomycin disk was measured and interpreted based on the manufacturer's instructions for *Neisseria gonorrhoeae*.

### PCR Amplification of Integrase Genes

Multiplex PCR was used to detect class 1, 2, and 3 integrons [4]. Whole-cell (genomic) DNA was obtained from each target strain by using a genomic DNA purification kit (SolGent, Daejeon, Korea) according to the manufacturer's instructions. PCR was performed with 50 ng of template DNA (genomic DNA), 2.5 μl of 10× Taq buffer, 0.5 μl of 10 mM dNTP mix, 20 pmol of each primer, and 0.7 U of Taq DNA polymerase (SolGent), in a total volume of 25 μl. Class 1, 2, and 3 integrons were amplified *via* the pre-denaturation of the reaction mixture for 5 min at 95°C; this was followed by 35 cycles for 30 sec at 95°C, for 40 sec at 52°C, and for 30 sec at 72°C, and a final elongation for 5 min at 72°C; these reactions were conducted in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Corp., Norwalk, CT., USA). The amplified products were separated *via* electrophoresis on 1.5% agarose gels containing ethidium bromide and visualized using a BioDoc-14TM Imaging system (UVP, Cambridge, UK).

### Characterization of Integrons

All the integron-positive isolates were subjected to PCR and sequencing assays using specific primers for the analysis of gene cassette arrays [18]. For sequencing, PCR products were purified with a PCR purification kit (SolGent) following the manufacturer's protocols. Sequencing was performed by a BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3730XL DNA analyzer (PE Applied Biosystems). DNA fragments (up to 1 kb in size) were sequenced using the overlapping PCR technique. The various DNA sequences were confirmed by using the BLAST paired alignment facility (http://blast.ncbi.nlm.gov).

## Results

### Enterobacteriaceae Isolates from Chicken Cecum

The bacterial species of Enterobacteriaceae isolated from chicken cecum were identified. A total of five different species composed of *E. coli* ($n = 72$), *Proteus mirabilis* ($n = 7$), *Proteus penneri* ($n = 4$), *Cronobacter* spp. ($n = 4$), and *Shigella dysenteriae* ($n = 3$) were obtained in our study. *E. coli* was the most prevalent species among chicken intestinal bacteria. The three genera (*Escherichia*, *Cronobacter*, and *Shigella*) were isolated from both Chungcheong and Gyeongsang provinces, but the genus *Proteus* was isolated from Chungcheong province only.

**Table 1.** Resistance of Enterobacteriaceae isolates from chicken cecum to 12 antimicrobial agents.

| Antimicrobial agents | No. (%) of resistant isolates | | | | | |
|---|---|---|---|---|---|---|
| | *E. coli* (*n* = 72) | *P. mirabilis* (*n* = 7) | *P. penneri* (*n* = 4) | *Cronobacter* spp. (*n* = 4) | *S. dysenteriae* (*n* = 3) | Total (*n* = 90) |
| Amikacin | 23 (31.9) | 1 (14.3) | 0 (0.0) | 3 (75.0) | 1 (33.3) | 29 (32.2) |
| Ampicillin | 54 (75.0) | 3 (42.9) | 3 (75.0) | 4 (100.0) | 2 (66.7) | 68 (75.6) |
| Cefotaxime | 39 (54.2) | 0 (0.0) | 2 (50.0) | 2 (50.0) | 1 (33.3) | 48 (53.3) |
| Ceftazidime | 1 (1.4) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 0 (0.0) | 2 (2.2) |
| Cephalothin | 54 (75.0) | 1 (14.3) | 1 (25.0) | 2 (50.0) | 3 (100.0) | 63 (70.0) |
| Chloramphenicol | 44 (61.1) | 3 (42.9) | 3 (75.0) | 3 (75.0) | 1 (33.3) | 60 (66.7) |
| Gentamicin | 11 (15.3) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (33.3) | 12 (13.3) |
| Kanamycin | 61 (84.7) | 3 (42.9) | 2 (50.0) | 2 (50.0) | 2 (66.7) | 74 (82.2) |
| Nalidixic acid | 71 (98.6) | 3 (42.9) | 3 (75.0) | 4 (100.0) | 3 (100.0) | 84 (93.3) |
| Spectinomycin | 55 (76.4) | 6 (85.7) | 3 (75.0) | 3 (75.0) | 2 (66.7) | 69 (76.7) |
| Streptomycin | 51 (70.8) | 2 (28.6) | 3 (75.0) | 4 (100.0) | 2 (66.7) | 67 (74.4) |
| Trimethoprim | 41 (56.9) | 3 (42.9) | 2 (50.0) | 2 (50.0) | 1 (33.3) | 54 (60.0) |

**Antimicrobial Susceptibilities of Bacterial Isolates**

The results of antimicrobial susceptibility tests for Enterobacteriaceae isolates are presented in Table 1. A total of 90 isolates identified in the current study showed the highest level of resistance in response to nalidixic acid (93.3%), and most of the antimicrobial agents also displayed relatively high resistance, except amikacin, ceftazidime, and gentamicin. Among the five species, more than 70% of *E. coli* and *Cronobacter* spp. isolates were resistant to six different antimicrobial agents tested. On the contrary, *P. mirabilis* isolates were the most susceptible species to all antimicrobial agents used in our study.

**Characterization of Integrons**

Among the 90 Enterobacteriaceae isolates tested, 46 (51.1%) carried integrase genes. Class 1 integrons were present in 40.3% (29/72), 42.9% (3/7), 75.0% (3/4), and 100.0% (4/4) of Enterobacteriaceae isolates that belonged to *E. coli*, *P. mirabilis*, *P. penneri*, and *Cronobacter* spp., respectively. Class 2 integrons were detected in eight of *E. coli* isolates and two of *Proteus* spp. isolates, of which three isolates carried both class 1 and 2 integrons. However, no class 3 integron was found in our isolates and no integrons were identified in all *S. dysenteriae* isolates.

To investigate the gene cassette arrays of integrons, class 1 and 2 integrons were amplified using specific primers followed by sequencing. Whereas all class 2 integrons did not contain gene cassettes, the class 1 integrons found in the present study carried seven different gene cassette arrays (Table 2). The genes encoding resistant determinants to aminoglycosides (*aadA1*, *aadA2*, and *aadA5*), trimethoprim (*dfrA1*, *dfrA12*, *dfrA17*, and *dfrA32*), lincosamides (*linF*), and

**Table 2.** Class 1 integron gene cassette arrays obtained from Enterobacteriaceae isolates.

| Cassette arrays | Numbers of isolates harboring class 1 integron (%) | | | | | |
|---|---|---|---|---|---|---|
| | *E. coli* (*n* = 72) | *P. mirabilis* (*n* = 7) | *P. penneri* (*n* = 4) | *Cronobacter* spp. (*n* = 4) | *S. dysenteriae* (*n* = 3) | Total (*n* = 90) |
| *aadA1* | 1 (1.4) | - | - | - | - | 1 (1.1) |
| *dfrA17* | 2 (2.8) | - | - | - | - | 2 (2.2) |
| *aadA1-dfrA1* | 19 (26.4) | - | - | 3 (75.0) | - | 22 (24.4) |
| *aadA5-dfrA17* | 5 (6.9) | - | - | 1 (25.0) | - | 6 (6.7) |
| *aadA2-linF* | 1 (1.4) | 1 (14.3) | 1 (25.0) | - | - | 3 (3.3) |
| *dfrA12-orfF-aadA2* | 1 (1.4) | - | - | - | - | 1 (1.1) |
| *dfrA32-ereA-aadA2* | - | 2 (28.6) | 2 (50.0) | - | - | 4 (4.4) |
| Total | 29 (40.3) | 3 (42.9) | 3 (75.0) | 4 (100.0) | 0 (0.0) | 39 (43.3) |

**Table 3.** Antimicrobial resistance rate (%) in class 1 integron-positive and -negative Enterobacteriaceae isolates.

| Report group | Antimicrobial agents | Class 1 integron-negative ($n$ = 51) | Class 1 integron-positive ($n$ = 39) | Total ($n$ = 90) |
|---|---|---|---|---|
| Aminoglycosides | Amikacin | 35.3 | 28.2 | 32.2 |
| | Gentamicin | 9.8 | 17.9 | 13.3 |
| | Kanamycin | 82.4 | 82.1 | 82.2 |
| | Spectinomycin | 68.6 | 87.2 | 76.7 |
| | Streptomycin | 74.5 | 74.4 | 74.4 |
| Cephems | Cefotaxime | 47.1 | 61.5 | 53.3 |
| | Ceftazidime | 2.0 | 2.6 | 2.2 |
| | Cephalothin | 76.5 | 61.5 | 70.0 |
| Folate pathway inhibitors | Trimethoprim | 41.2 | 84.6 | 60.0 |
| Penicillins | Ampicillin | 60.8 | 94.9 | 75.6 |
| Phenicols | Chloramphenicol | 56.9 | 79.5 | 66.7 |
| Quinolones | Nalidixic acid | 92.2 | 94.9 | 93.3 |

erythromycin (*ereA*) were harbored in the class 1 integrons in Enterobacteriaceae isolates from chicken cecums.

Next, we compared the resistance rate of antimicrobial agents between integron-negative and integron-positive isolates to investigate whether there was any correlation between harboring integrons and antimicribial resistance (Table 3). Isolates containing class 1 integrons had a higher incidence of resistance to ampicillin, chloramphenicol, spectinomycin, trimethoprim, and gentamicin, but a lower incidence of resistance to nalidixic acid.

## Discussion

Antimicrobial resistance has become serious public health threats throughout the world, and therapeutic options for several infectious diseases are currently limiting with a presence of multidrug-resistant bacteria. The long-term and widespread use of antimicrobial agents contributes to the selection of antimicrobial-resistant bacteria. In our present study, we aimed to investigate the antimicrobial resistance rates and genetic determinants of Enterobacteriaceae isolates from food animals in Korea. We showed that Enterobacteriaceae isolates from chicken cecums were highly resistant to common antimicrobial agents, such as ampicillin, cephalothin, kanamycin, nalidixic acid, spectinomycin and streptomycin. Specifically, the overall antimicrobial resistance rates were higher in *E. coli* isolates compared with commensal *E. coli* isolates from humans. *E. coli* isolates from chicken showed a resistance rate the ranged between 56.9% and 84.7% to ampicillin, chloramphenicol, kanamycin, streptomycin, or trimethoprim, whereas *E. coli* isolates from humans displayed between 3.6% and 36.5% to the same antimicrobial agents [12].

To our knowledge, our study is the first to isolate *Cronobacter* spp. (formerly *Enterobacter sakazakii*) from poultry in Korea. These strains are primary pathogens for necrotizing enterocolitis and meningitis in infants, and it has been reported that they were contaminated in infant formula and a wide range of foods and environments, causing food-borne diseases [9]. Interestingly, all the *Cronobacter* spp. isolates identified in our study have shown to possess class 1 integron.

Integrons, especially class 1 and 2, have contributed to disseminate antimicrobial resistance genes mainly in many gram-negative bacteria. Therefore, the present study attempted to determine the frequency of various class 1 and 2 integrons among Enterobacteriaceae isolates from chicken cecums. Approximately, half of the Enterobacteriaceae isolates (51.1%) harbored class 1 integrons, where 40.3% of *E. coli* was shown to possess class 1 integron and the frequencies were even higher in *Proteus* and *Cronobacter* spp. The incidence of *E. coli* isolates containing class 1 integrons was comparable to that of *E. coli* isolates from large intestine of poultry in Korea (42.3%), but lower than from chicken feces (54.5%) [9, 3]. However, the frequencies (40.3%) were still considerably higher against healthy human stool (11.4%) or clinical specimens (30.8%) [9].

In this study, seven gene cassette arrays were identified in the Enterobacteriaceae isolates. *aadA1-dfrA1* was the most prevalent class 1 integron detected in *E. coli* and *Cronobacter*

spp. isolates, followed by *aadA5-dfrA17*. However, the two gene cassette arrays were not detected in isolates of genus *Proteus*, which harbored *aadA2-linF* or *dfrA32-ereA-aadA2*. It is notable that *dfrA32-ereA-aadA2* was identified only in *Proteus* isolates, which have not been previously reported in Korea. *dfrA32-ereA-aadA2* has been found in *P. mirabilis* and *Laribacter hongkongensis* in China [5]; however, we identified this integron in *P. penneri* as well as *P. mirabilis*.

In addition, the *aadA* gene (streptomycin and specitinomycin resistance) was the most prevalent gene cassette of the class 1 integrons, followed by the *dfrA* gene (trimethoprim resistance): the *aadA* and *dfrA* genes were detected in six and five class 1 integrons identified from Enterobacteriaceae isolates, respectively. These were also frequently present in class 1 integrons isolated from humans, poultry, and swine in Korea [12]. Moreover, the isolates harboring the *aadA* and/or *dfrA* gene showed a higher resistance rate to spectinomycin and/or trimethoprim in the present study (Table 3). Previously, higher resistance percentages have been observed against several antimicrobial agents in integron-positive than integron-negative isolates [17]. However, all the isolates from our study were highly resistant to streptomycin, regardless of containing integrons (Table 3). Streptomycin resistance is usually integron-mediated, but it cannot be illustrated by one antimicrobial resistance mechanism. Therefore, further investigations are necessary in the future to confirm about streptomycin resistance mechanisms that are not direly related to integrons. Moreover, all the class 1 integrons identified did not contain ampicillin or chloramphenicol resistance genes, but the isolates harboring class 1 integrons showed higher resistance rates to these antimicrobial agents than integron-negative isolates. Therefore, these results might imply the presence of another horizontal gene transfer platform associated with class 1 integrons. This suggestion also agreed with a previous study that nonclassical class 1 integrons containing *cmlA* was related to chroramphenicol resistance [17].

In conclusion, we confirmed that class 1 integrons are widely distributed in Enterobacteriaceae isolates from chickens and directly contribute to the resistance to diverse antimicrobial agents. Accordingly, our study suggests that the continuous investigation of integron gene cassette arrays will provide useful information regarding antimicrobial resistance mechanisms.

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