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High incidence of multidrug resistance and class 1 and 2 integrons in *Escherichia coli* isolated from broiler chickens in South of Iran

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Abstract

The objective was to investigate the multidrug resistance and presence of class 1 and 2 integrons in 300 *Escherichia coli* isolates obtained from 20 broiler farms during three rearing periods (one-day-old chicks, thirty-day-old chickens, and one day before slaughter) in Fars, South Iran. Results showed that 81.00%, 82.00%, and 85.00% of isolates were multidrug-resistant on the first day, thirty-day-old chickens, and one day before slaughter, respectively. Multidrug-resistant *E. coli* isolates were further examined for the presence of class 1 and 2 integrons using PCR assay. The existence of class 1 integron-integrase gene (*intI1*) was confirmed in 68.40%, 72.70%, and 60.90% of multidrug-resistant isolates from stage 1, stage 2, and stage 3 of the rearing period, respectively. The frequency of class 2 integron-integrase gene (*intI2*) during the first to the third stage of sampling was 2.60%, 25.50%, and 30.40%. Also, sequence analysis of the cassette arrays within class 1 integron revealed the presence of the genes associated with resistance for trimethoprim (*dfr*A), streptomycin (*aad*A), erythromycin (*ere*A), and *orf*F genes. The results revealed that percentages of antimicrobial resistance in *E. coli* isolates were significantly higher in the middle and end stages of the rearing period. In conclusion, widespread dissemination of class 1 integrons in all three stages and rising trends of class 2 integrons existence in *E. coli* isolates during the rearing period of broiler chickens could exacerbate the spread of resistance factors among bacteria in the poultry industry. Future research is needed to clarify its implication for human health.

Introduction

Over the past decades, the rising trend of antimicrobials as growth promoters and prophylactic and therapeutic agents in the poultry industry has caused a challenging issue of increasing bacterial antimicrobial resistance.[1] The broad application of antimicrobials in chickens has increased the risk of bacterial resistance, and the administration of more than one antimicrobial during the rearing period of chickens may cause the dissemination of antimicrobial resistance, especially in gram-negative bacteria such as *Escherichia coli*.[2] Selective pressure resulting from the use of antimicrobials in the poultry industry is found to be associated with multiple-drug resistance (MDR) in both commensal and pathogenic *E. coli*.[3] This phenomenon is not only resulted from the natural ability of bacteria to survive and proliferate to more numbers but also related to horizontal gene transfer through plasmids.[4]

Multidrug resistance in enteric organisms such as *E. coli* has been recognized to be related to integrons.[5] Integrons are bacterial genetic platforms that can increase the uptake and gene expression in their gene cassettes.[6] Integrons classification is generally based on the sequence of integrase protein that gives them recombination ability. Up to date, four general classes of integrons have been recognized and distinguished, out of which most of the studies have been done on class 1, and 2.[5] Class 1 integrons are widely disseminated among Gram-negative bacteria in humans and animals. This class is the most common class of Integrons in clinical isolates and so is called clinical integrons.[7]

More than 130 different gene cassettes have been identified in different integrons classes, which cause

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resistance to almost all antimicrobials.[8] Integron gene cassettes that have been identified to date are typically encoding antimicrobial resistance factors and are known as Resistance Integrons (RIs) or Multi-drug Resistance Integrons (MRIs).[9] Adjacent resistance genes on integrons have been stated to encode MDR and get involved in transferring this situation in bacteria.[10]

The presence of integrons has been reported in multidrug-resistant commensal and pathogenic *E. coli* isolated from chicken farms worldwide.[11-18] In Iran, preliminary studies showed a high prevalence of phenotypic and genotypic resistance to fluoroquinolones and tetracyclines in *E. coli* isolated from broiler chickens during different stages of a rearing period.[19,20] Accordingly, this study was aimed to determine the incidence of MDR for intestinal *E. coli* isolated from broiler chickens during the rearing period and distribution of class 1 integron-integrase gene (*intI1*) and class 2 integron-integrase gene (*intI2*) in these isolates.

Materials and Methods

Sampling. Samples were taken from 20 broiler chicken flocks from different farms located in Fars province, South of Iran. Sampling was done in three stages of the rearing period: one-day-old broiler chicks on arrival at the farms, 30 days of age, and one day before slaughter (42-47 days old). This study complied with the Ethical Principles in Animal Research, approved and performed in accordance with the ethical standards of the Committee for Animal Experiments of School of Veterinary Medicine, Shiraz University (IACUC no: 4687/63). The research was conducted under the permission of a farm owner who gave informed consent for the study to be carried out. Five pooled cloacal swabs were taken from each farm in glass tubes containing tryptic soy broth (TSB) medium (Merck, Darmstadt, Germany) and instantly transferred to the laboratory.

Isolation and identification of *E. coli*. Tryptic soy broth medium contents were cultured on MacConkey agar (Merck) plates and incubated overnight at 37.00 ˚C. A pink colored distinct colony from each plate was used to culture on the Eosin Methylene Blue (EMB) agar (Merck) plates and incubated for 24 hr at 37.00 ˚C. Greenish metallic colonies on EMB agar were considered *E. coli* and identified using standard biochemical tests (Gram stain, oxidase test, TSI test, indole test, citrate test, methyl red and Voges-Proskauer tests, and urea agar test).[21] Confirmed isolates were deposited in TSB medium with 30.00% glycerol at –70.00 ˚C.

Antimicrobial susceptibility test. *E. coli* isolates were tested for susceptibility to nalidixic acid (NAL, 30.00 µg), flumequine (FM, 30.00 µg), ciprofloxacin (CIP, 5.00 µg), enrofloxacin (ENR, 5.00 µg), norfloxacin (NOR, 10.00 µg), ampicillin (AM, 10.00 µg), furazolidone (FR, 100 µg), gentamicin (GM, 10.00 µg), neomycin (N, 30.00 µg), lincospectin (LP, 200/15.00 µg), tetracycline (TET, 30.00 µg), oxytetracycline (T, 30.00 µg), chloramphenicol (C, 30.00 µg), florfenicol (FF, 30.00 µg), erythromycin (E, 15.00 µg), streptomycin (S, 10.00 µg), and trimethoprim-sulfamethoxazole (SXT, 23.75/1.25 µg) on Mueller-Hinton agar (Merck) by the disc diffusion method (antibiotic discs: PadtanTeb, Tehran, Iran) as described in the National Committee for Clinical and Laboratory Standards.[22,23] The *E. coli* ATCC 25922 reference strain was used as the quality control.

The presence of multiple resistance. Recently standardized criteria developed by European Centre for Disease Prevention and Control (ECDC) were used to define Multidrug-resistant (MDR) *E. coli* isolates (non-susceptible to ≥3 classes of drugs), extensively drug-resistant (XDR) *E. coli* isolates (non-susceptible to all but 1 or 2 classes of drugs), and pandrug-resistant (PDR) *E. coli* isolates (non-susceptible to all antimicrobial classes) considering the following antimicrobial categories: Aminoglycosides, penicillins, folate pathway inhibitors, phenicols, tetracyclines, and fluoroquinolones.[24]

PCR amplification of integrase genes. DNA extraction was done using the boiling method.[25] Integrons class 1 and 2 were investigated among 139 multidrug-resistant *E. coli* isolates (38 isolates for stage one, 55 and 46 isolates for stage two and three, respectively), and, consequently, gene cassettes were explored in isolates that were positive for the *intI1* gene, using PCR and direct sequencing. The primers for amplifying the *intI1* gene were intM1-U (5′-ACGAGCGCAAGGTTTCGGT-3′) and intM1-D (5′-GAAAGGTCTGGTCATACATG-3′), which produced a 565 bp fragment. The primer was paired to amplify the *intI2* gene (403 bp) was intM2-U (5′-GTGCAACGCATTTTG CAGG-3′) and intM2-D (5′-CAACGG AGTCATGCAGATG-3′).[26] Also, to identify the gene cassette element(s) in class 1, the integron, we used in-F (5′-GGCATACAAGCAGCAAGC-3′) as the forward primer and in-B (5′-AAGCAGACTTGAC CTGAT-3) as the reverse one.[27] The PCR reaction (20.00 µL) was performed in 10.00 mM Tris-HCl, pH = 8.30-8.80, 50.00 mM KCl, 1.50 mM MgCl2, 0.20 mM dNTPs, 10.00 pmoL of forward and reverse primers (Gen Fanavaran Co., Tehran, Iran), 2.00 U Taq DNA polymerase, and 2.00 µL (~40.00 ng) of the DNA extract as template.

Statistical analysis. Chi-square (χ^2) test was performed to evaluate the susceptibility or resistance against different antimicrobials and the frequency of integrase genes of class 1 and 2 integrons in *E. coli* isolated during the three stages of sampling. This test was also used to calculate the association between antimicrobial resistance profile and integron existence. The results were statistically analyzed using the SPSS statistical software (version 16.0; SPSS Inc., Chicago, USA). Differences among means with $p < 0.05$ were accepted as statistically significant.

Results

The antimicrobial susceptibility test results during the rearing period 1, 2, and 3 are presented in Table 1. The antimicrobial susceptibility test showed high-level resistance to erythromycin and streptomycin in all three stages. There were no significant differences among the stages in antimicrobial resistance ($p > 0.05$). However, resistance against other antimicrobials except ampicillin was significantly lower in day-old chicks than the middle and last days of the rearing period ($p < 0.05$). Ampicillin showed a different trend in contrast to other antimicrobials, where ampicillin resistance was significantly higher in day-old chicks ($p < 0.05$).

Overall, 82.60% of isolates were MDR and 48.00% of *E. coli* isolates were extensively drug resistant (XDR), and 2.60% of isolates were pandrug-resistant (PDR). The highest percentage of MDR isolates (85.00%) was seen in stage 3 (a day before slaughter). The detailed description of three stages of rearing period is as follows: Stage 1 (n = 100), 81.00% MDR, 54.00% XDR and 4.00% PDR; stage 2 (n = 100), 82.00% MDR, 45.00% XDR and 2.00% PDR; stage 3 (n = 100), 85.00% MDR, 45.00% XDR and 2.00% PDR.

After determining MDR isolates, all the resistant isolates to at least ten antimicrobials of the seventeen inspected antimicrobials were chosen for the detection of class 1 and class 2 integrons. According to this procedure, 139 MDR isolates were selected from three stages: 38 isolates from one-day-old chicks, 55 from 30-day-old chickens, and 46 isolates from ready to slaughter chickens. The frequencies of *intI1* and *intI2* genes among the three stages of sampling are shown in Table 2. In general, from all of the 139 isolates, 67.60% were positive for *intI1*, 20.90% for *intI2,* and 8.60% for both integrase 1 and 2 genes (Fig. 1A). Results showed that the presence of the *intI1* gene among *E. coli* isolates from three stages were not significantly different ($p > 0.05$). However, *intI2* gene was significantly lower in one-day-old chicks than the two later stages of the rearing period ($p < 0.05$).

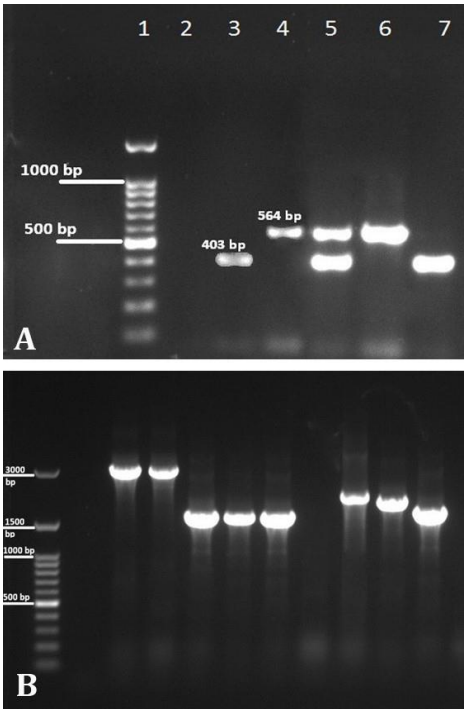

Fig. 1. A) PCR amplification of integrase 1 and 2 genes in *E. coli* isolated from broiler chickens. Lane 1: DNA marker, Lane 2: Negative control, Lane 3: Positive control of integrase 2, Lane 4: Positive control of integrase 1, Lane 5: Isolate positive for co-existence of both integrase genes, Lane 6: Isolate positive for integrase 1 gene, Lane 7: Isolate positive for integrase 2; **B)** PCR amplification of genes cassette in class 1 integrons. Bands with different sizes (~3200 bp, 1586 bp, 2097 bp, 1913 bp, and 1664 bp) harbor different resistance genes.

Table 1. Antibiotic susceptibility test of *E. coli* isolates during a rearing period of broiler chickens (Stage1: day-old chicks, Stage 2: 30-day-old, and Stage 3: A day before slaughter).

Antimicrobials	Stage 1 (n=100)			Stage 2 (n=100)			Stage 3 (n=100)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Nalidixic acid	20.00	3.00	77.00	8.00	0.00	92.00	0.00	0.00	100.00
Flumequine	22.00	2.00	76.00	8.00	0.00	92.00	0.00	0.00	100.00
Ciprofloxacin	58.00	5.00	37.00	18.00	5.00	77.00	12.00	7.00	81.00
Enrofloxacin	24.00	30.00	46.00	15.00	7.00	78.00	3.00	7.00	90.00
Norfloxacin	55.00	6.00	39.00	17.00	5.00	78.00	6.00	12.00	82.00
Ampicillin	23.00	31.00	46.00	54.00	11.00	35.00	52.00	17.00	31.00
Furazolidone	74.00	1.00	25.00	47.00	1.00	52.00	28.00	0.00	72.00
Gentamicin	98.00	0.00	2.00	88.00	6.00	6.00	85.00	6.00	9.00
Neomycin	1.00	43.00	56.00	2.00	25.00	73.00	0.00	16.00	84.00
Lincospectin	63.00	20.00	17.00	49.00	23.00	28.00	57.00	10.00	33.00
Tetracycline	33.00	0.00	67.00	7.00	3.00	90.00	2.00	4.00	94.00
Oxytetracycline	33.00	0.00	67.00	6.00	0.00	94.00	4.00	2.00	94.00
Chloramphenicol	55.00	0.00	45.00	38.00	5.00	57.00	24.00	0.00	76.00
Florfenicol	83.00	1.00	16.00	51.00	5.00	44.00	74.00	5.00	21.00
Erythromycin	2.00	0.00	98.00	0.00	1.00	99.00	0.00	1.00	99.00
Streptomycin	2.00	53.00	45.00	0.00	48.00	52.00	1.00	64.00	35.00
Trimethoprim-sulfa	64.00	0.00	36.00	20.00	3.00	77.00	17.00	3.00	80.00

S: susceptible, I: intermediate, R: resistant.

Table 2. The number and percentage of integrase 1 (*intI1*), integrase 2 (*intI1*) genes, and co-existence of *intI1* and *intI2* genes in MDR *E. coli* isolated during a rearing period of broiler chickens (stage1: one-day-old chicks, stage 2: 30-day-old and stage 3: Aday before slaughter).

Stages	Isolates	*intI1* (%)	*intI2* (%)	*intI1* + *intI2* (%)
1	38	26 (68.40)[a]	1 (2.60)[a]	1 (2.60)[a]
2	55	40 (72.70)[a]	14 (25.50)[b]	7 (12.70)[a]
3	46	28 (60.90)[a]	14 (30.40)[b]	4 (8.70)[a]

[ab] Columns with different superscripts have significant differences ($p < 0.05$).

Five different class 1 integron gene cassette arrays, classified as type I–V, were identified in the class1 integron positive isolates (Fig. 1B). Four different genes were identified, including dihydrofolate reductase (*dfr*A), amino-glycoside adenylyltransferase (*aad*A), erythromycin esterase (*ere*A), and a hypothetical protein (*orf*F), (Table 3).

Discussion

High rates of antimicrobial resistance were found in this study, even in *E. coli* isolated from one-day-old chicks. It has already been shown that antimicrobial-resistant *E. coli* isolated from broiler chicks could be inherited vertically from chicken breeders.[28,29] Therefore, anti-microbial resistance rates in a non-treated broiler farm could be affected by antimicrobial treatments in their broiler breeders.[30] Vertical transmission from broiler breeders and the acquisition of antimicrobial resistance bacterial isolates from hatcheries could introduce antimicrobial resistant bacterial clones to broiler farms.[31] Antimicrobial resistance could increase from one-day- old chicks to ready to slaughter chickens, independent of the use of antimicrobials.[32,33] However, selective pressure due to antimicrobial use during the rearing period could intensely increase the rate of antimicrobial resistance.[34-37] Our previous work demonstrated that antimicrobial use in a rearing period was the most effective risk factor for rising fluoroquinolone resistance during a rearing period of broilers.[20]

In the present study, MDR was high among *E. coli* isolates even in one-day-old chicks, and the rising trend of this resistance was not statistically different during the rearing period. The high rate of multiple drug resistance in one-day-old chicks could be a direct consequence of high resistance against some of the antimicrobials in these chicks. It has been publicized that resistance to a specific antimicrobial could dramatically shift their microbial antibiogram to a multidrug resistance profile even in the early days of chicken life.[34] High rates of multidrug resistance persisted steadily in the *E. coli* isolated in our study during the middle and last day of the rearing period so that 85.00% of isolates from ready to slaughter chickens showed multidrug resistance. This too high MDR rate in *E. coli* isolated from pre-slaughter chickens could be challenging for human health because there is some evidence on transmission of multidrug-resistant *E. coli* clones, plasmids, and other transmissible elements such as integrons from poultry to humans.[38-40]

The high incidence of the *intI1* gene (67.60%) was found among MDR cloacal *E. coli* isolates in our study, while only 20.90% of isolates had *the intI2* gene. Higher frequency of class 1 compared to class 2 integrons were consistent with the previous studies in commensal and pathogenic *E. coli* isolated from poultry, especially in chickens.[15,41-44] In contrast, there are infrequent reports on a slightly higher incidence of *intI2* gene in *E. coli* isolated from turkeys.[45]

Table 3. Size and contents of gene cassettes and antibiotic resistance profile of sequenced MDR *E. coli* isolates.

Sequenced samples	Cassette size (bp)	Gene cassettes	Resistance phenotype
1	1586	*dfr*A1, *aad*A1	LP, TET, C, S, FM, SXT, E, NOR, ENR, T, NAL, CIP, FR, FF, N
2	1586	*dfr*A1, *aad*A1	LP, TET, S, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FF, N
3	1664	*dfr*A17, *aad*A5	TET, C, S, FM, SXT, E, NOR, ENR, T, NAL, CIP, FR, N
4	1913	*dfr*A12, *orf*F, *aad*A2	LP, TET, C, S, GM, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FR, N
5	1586	*dfr*A1, *aad*A1	LP, TET, C, S, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FR, FF, N
6	1664	*dfr*A17, *aad*A5	TET, C, S, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, N
7	1586	*dfr*A1, *aad*A1	LP, TET, C, S, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FR, FF, N
8	~ 3200	*dfr*A17, *ere*A1, *aad*A2	TET, C, S, GM, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FR, N
9	1913	*dfr*A12, *orf*F, *aad*A2	LP, TET, C, S, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FR, N
10	1586	*dfr*A1, *aad*A1	TET, C, S, GM, FM, SXT, E, NOR, ENR, T, NAL, FR, FF, N
11	2097	*dfr*A5, *ere*A2	LP, TET, C, S, FM, SXT, E, NOR, ENR, T, NAL, CIP, FR, FF, N
12	1913	*dfr*A12, *orf*F, *aad*A2	LP, TET, C, S, FM, SXT, E, NOR, ENR, T, NAL, CIP, FR, N
13	1586	*dfr*A1, *aad*A1	LP, TET, C, S , FM, SXT, E, NOR, ENR, T, NAL, CIP, FR, FF, N
14	2097	*dfr*A5, *ere*A2	TET, C, S, FM, SXT, E, NOR, ENR, T, NAL, CIP, FR, FF, N
15	1586	*dfr*A1, *aad*A1	TET, S, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FF, N
16	~ 3200	*dfr*A17, *ere*A1, *aad*A2	LP, TET, C, S, FM, SXT, E, AM, NOR, ENR, T, NAL, CIP, FR, FF, N

NAL: Nalidixic acid, FM: Flumequine, CIP: Ciprofloxacin, ENR: Enrofloxacin, NOR: Norfloxacin, AM: Ampicillin, FR: Furazolidone, GM: Gentamicin, N: Neomycin, LP: Lincospectin, T: Tetracycline, TET: Oxytetracycline, C: Chloramphenicol, FF: Florfenicol, E: Erythromycin, S: Streptomycin, SXT: Trimethoprim-sulfamethoxazole.

Other investigations are dealing only with the presence of class 1 integrons in *E. coli* isolated from poultries of USA,[46] China,[11] Hungary,[12] Korea,[13] and Belgium,[47] for which class 1 integrons were positive for 63.00%, 59.00%, 41.00%, 39.60%, and 21.60% of isolates, respectively. Our study results showed a high incidence of *intI1*gene among *E. coli* isolated from three stages of the rearing period, which was not significantly different between these stages. On the other hand, a significantly higher incidence of the *intI2* gene was found in the middle and last days of the rearing period. These findings could emphasize the role of the *intI2* gene in triggering, more excellent antimicrobial resistance during the second and later stages of sampling in this study.

Sequence analysis of *intI1* cassette arrays showed different types of antimicrobial resistance genes for three antimicrobials family consisting of macrolides (erythromycin), aminoglycosides (streptomycin), and folic acid synthesis inhibitors (trimethoprim), and one more gene (*orf*F), causing no known antimicrobial resistance so far. These resistance genes showed five different cassette arrangements. Soufi *et al*. studied 166 *E. coli* isolates recovered from poultry meat in Tunisia and showed that *aad*A (types 1, 2, and 5), *dfr*A (types 1, 12, 14, and 17), and *orf*F in five different cassette arrays were associated with class 1 integrons.[48] Another study in China showed that 59.00% of *E. coli* isolates recovered from broiler chickens had class 1 integrons, which streptomycin and trimethoprim resistance (*dhfr*1*, aad*A1*, dhfr*17*, aad*A2*, dhfr*13*)* were harbored in the variable zone of them.[11] Cavicchio *et al*. investigated class 1 and class 2 integrons in avian pathogenic *E. coli* from poultry in Italy and showed that *aad*A1 and the combinations *of aad*A1-*dfr*A1 and *dfr*A1-*aad*A1 genes were the most common cassette arrays in class 1 integrons.[15] In our study, the combination of *dfr*A1 and *aad*A1 was the most common gene cassettes in class 1 integrons of cloacal *E. coli* isolates. Similar results were shown by Yang *et al*., Kim *et al*. and Cocchi *et al*. for avian pathogenic *E. coli* isolates. [11,13,49]

In conclusion, the present study results revealed high percentages of multi-drug resistance in commensal *E. coli* isolates from broiler chickens with the widespread dissemination of class 1 and the rising trend of class 2 integrons existence in these *E. coli* isolates during the rearing period of broiler chickens. These trends could exacerbate the spread of resistance factors among bacteria in the poultry industry and a rising global threat for public health in slaughtered chickens.

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Conflict of interest

The authors declare no conflicts of interest and no financial, personal, or other relationships with other people or organizations.

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