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Original Research

Molecular analysis of integron gene cassette arrays associated multi-drug resistant Enterobacteriaceae isolates from poultry

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Abstract: The study investigated 110 Enterobacteriaceae isolates from broiler chickens isolated from Sharkia poultry farms and analyzed the isolates antimicrobial resistance and the presence of integrons as a potential basis for this resistance. Antibiotic susceptibilities against 12 different antibiotics were determined by the disk diffusion method. Prevalences and classes of integrons were then detected in multi-drug resistant (MDR) strains using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) followed by sequencing of the variable parts. Fifty-three isolates were MDR (resistant to three or more antimicrobial agents). High resistance was detected for rifamycin (82.7%), erythromycin (67.2%), and amoxicillin-clavulanic acid (63%). Classes 1 and 2 integrons were detected in 38 of 53 MDR Enterobacteriaceae isolates of which the most common were *Salmonella species* (n=19), followed by *Escherichia coli* (12), *Klebsiella pneumoniae* (3), *Proteus species* (3), and *Citrobacter freundii* (1). Three isolates only harbored class 1 integrons while the remaining 35 isolates carried class 2. All class 1 integron positive isolates exhibited the same gene cassettes arrangements: 1.) *dfrA12-orfF–aadA27* (1.6 kbp); 2.) *aadA23* (1.0 kbp); and 3.) *dfrA15* (0.8 kbp). Moreover, four different gene cassettes were identified within class 2 integrons: 1.) *dfrA1-sat2-aadA30* (2 kbp) in all isolates; 2.) *sat2-aadA1* (1.7 kbp) in only one isolate; 3.) *catB2* (0.9 kbp) in four isolates; and 4.) a new variant of *sat2* (0.65 kbp) in three isolates. Efforts should be made to introduce surveillance programs for monitoring antimicrobial resistance that could potentially be transmitted from broiler chickens to human via integrons.

Key words: Poultry; Enterobacteriaceae; Multidrug resistance; Integrons; RFLP; Gene cassettes.

Introduction

Antimicrobial agents are extensively used to treat or prevent bacterial diseases in the poultry industry, but the prevalence of multidrug-resistant (MDR) bacteria (i.e., resistant bacteria to three or more antimicrobial categories) has become worrisome and represented a great hazard to public health. In particular, antimicrobial agents are added to the feed and water or injected into chicks with a veterinary prescription on individual farms, resulting in an increase of antimicrobial-resistant intestinal bacteria that may be transferred to humans through food chains (1).

The genera belonging to Enterobacteriaceae inhabit intestinal tracts of poultry and are common pathogens causing serious 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 (2). Therefore, these facts confirm the occurrence of serious risks from the spreading of these resistance genes among bacterial species, and ultimately, to humans.

The genetic mechanisms of generation and transmission of antimicrobial resistance have become a significant topic in order to enhance the propagation of the multidrug-resistant bacteria (3).

Integrons are one of the important genetic platforms for antimicrobial resistance that are located either on plasmids or on the bacterial chromosome and may be part of transposons and strongly correlated with MDR in Enterobacteriaceae (4).

Integrons are a system of gene capture and expression in the form of cassettes and composed of an integrase gene (*intI*), encodes a site-specific recombinase; an adjacent attachment site, acts as a recipient for resistant genes; and a specific promoter responsible for the expression of any suitably integrated gene 5. Several classes of integrons have been described according to the sequence of *intI* gene. Classes 1 and 2 are well identified and involved in antibiotic resistance (5).

Class 1 integron is the most prevalent type in avian isolates of Enterobacteriaceae and has a 5′ conserved segment and a 3′ conserved segment. The 5′ conserved segment composed of the integrase gene (*intI1*), the promoters (Pint, Pc) and a recombination site (attI). The 3′ conserved segment usually contains the *qac*E gene, the *sul*1 gene, and a variable region (6). Class 2 integrons are associated with the Tn7 transposons, whose transposition activity is directed at specific attachment sites on chromosomes or plasmids and its sequence of its integrase gene (*intI*2) is identical to that of *intI*1, but it is ended prematurely and does not encode a complete functional integrase (7). Although the class 2 integrons share their cassettes pool with the class 1 integrons, they are distinguished by divergent integrase sequences (8).

Therefore, the aim of our study was to demonstrate

the assessing and distribution of class 1 and class 2 integrons and the patterns of the gene cassettes they carry in Enterobacteriaceae isolated from commercial poultry farms in Egypt in correlation with their resistance phenotypes to confront and overcome this resistance.

Materials and Methods

Sampling and isolates characterization

A total of 300 samples of liver, heart, ovary, kidney, and spleen were aseptically collected from 100 freshly dead and diseased broiler chickens from different localities in Sharkia Province, Egypt, during the period of June 2015 to January 2016.

All samples were subjected to conventional methods for isolation and identification of enterobacterial members (9) and were further identified with API20E identification kits (BioMérieux, Maryl'Etoile, France) and serotyped in the Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt using commercial antisera (Difco, Detroit, MI, USA) according to the manufacturer's instructions.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility test was performed using the disk diffusion method on Mueller-Hinton Agar (OXOID), according to the procedure recommended by the Clinical and Laboratory Standards Institute (10). All isolates were tested for various routine antimicrobial drugs (OXOID), the tested antibiotics and their concentrations on µg/disk were as following: streptomycin (S; 10), gentamicin (CN;10); kanamycin (K; 30), amoxicillin-clavulanic acid (AMC;20/10), ceftriaxone (CRO;30), rifamycin (RF;30), doxycycline (DO;30), chloramphenicol (C;30), sulfamethoxazole-trimethoprim (SXT;25), erythromycin (E;15), ciprofloxacin (CIP;5), and imipenem (IPM;10). The inhibition zones, in millimeters, were measured in duplicate and scored as sensitive, intermediate, and resistant categories in accordance with the critical breakpoints recommended by the Clinical and Laboratory Standards Institute.

PCR screening of integrons

Plasmid DNAs of bacterial isolates were extracted using QIAprep Spin Miniprep Kits (Qiagen, UK).

Integrons were detected by PCR with the degenerate primers designed to hybridize conserved regions of encoded integrase genes *intI*1, *intI*2, hep35 (5′-TGCGGGTYAARGATBTKGATTT-3′), and hep36 (5′-CARCACATGCGTRTARAT-3′) give a PCR product of 491 bp (11).

RFLP for Differentiation of Class 1 and Class 2 Integrons

Digestion of PCR Product Using *Rsa* I restriction enzyme 11, PCR products were subjected to digestion with *Rsa* I as follow, 10 µl of the amplified gene segment, 2 µl of the l0 x buffer supplied, 1µl of the enzyme and complete reaction with sterile water to attain the final volume of 20 µl, which was incubated for 3 hs at 37◦C. Then, the total volume was loaded onto the agarose gel. After digestion, integrase 1 gave rise to one fragment of 491 bp, while integrase 2 gave rise to two fragments of 334 and 157 bp, respectively.

Amplification of Gene Cassettes of Class 1 and Class 2 Integrons

Class 1 integron cassette structures were amplified using hep58 (5′-TCATGGCTTGTTATGACTGT3-3′) and hep59 (5′-GTAGGGCTTATTATGCACGC-3′) which bind 3′-CS and 5′-CS conserved segments, respectively. Class 2 integron cassette regions were amplified using hep74(5′-CGGGATCCCGGACGGC ATGCACGATTTGTA-3′), which binds to attI2 and hep51 (5 -GATGCCATCGCAAGTACGAG-3′), which binds to *orfX* situated downstream of the cassette region within Tn7 11. PCR was performed for 30 cycles; each cycle consisted of 94◦C for 30 s, 55◦C for 30 s and extension at 72◦C for 45 s for amplification of the *integrase* genes, or 4min for amplification of the cassette region. Amplification cycles were performed with DNA thermal cycler (Biometra, Germany), as mentioned elsewhere (12). For each batch of PCR reactions, positive and negative controls were included. The positive control was an isolate confirmed as integrase positive by DNA sequencing. PCR products were analyzed in parallel with a DNA MW-marker (Fermentas) by electrophoresis on 2% agarose gel.

DNA Sequencing

PCR products were purified using QIAquick PCR Product extraction kit. (Qiagen, Valencia). Big Dye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then it was purified using Centrisep spin column. DNA sequences were obtained from Applied Biosystems 3130 genetic analyzer (California, USA) and Alignment of the nucleotide sequences was performed by the use of *Bioedit program*, then translation to amino acid sequences was performed using the *ExPASy (Expert Protein Analysis System*) Translate Tool *(http://us.expasy.org/, Swiss Institute of Bioinformatics SIB, Geneva, Switzerland*).

Results

Bacterial Isolates

In this study, a total of 110 bacterial isolates belonging to the family Enterobacteriaceae were obtained from 100 examined broiler chickens. These included *Salmonella* (45), *E. coli* (40)*, Proteus* (10)*, Klebsiella* (8)*, Citrobacter* (4) and *Enterobacter* species (3).

Serotyping of *Salmonella* species revealed that *S. Typhimurium* was the most prevalent serotype (46.15%) followed by *S. Enteritidis* (23.07%), *S. Virchow* (11.5%), *S. Birkenhead* and *S. Kentucky* (7.69% each) and *S. Montevideo* (3.84%). Concerning to *Escherichia coli,* five serotypes were obtained, including O158, which accounted for 56.25% of total *E. coli* isolates. Other serotypes isolated (43.75%) were O111, O26, O27, and O78.

Furthermore, all obtained Citrobacter isolates were serotyped as *C. freundii*. *Proteus mirabilis* and *Proteus vulgaris* were detected with percentage 90% and 10%, respectively. Additionally, two serotypes of *Enterobacter* species were obtained as *Enterobacter hafnia* (66.6%) and *Enterobacter cloacae* (33.4%).

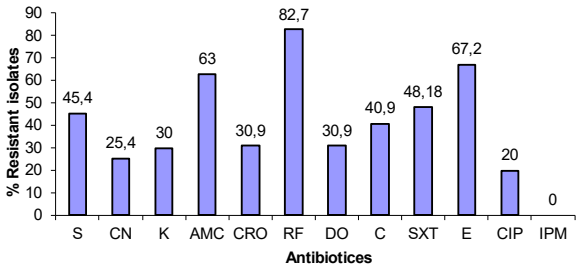

Figure 1. Percentage of antimicrobial resistance of Enterobacteriaceae isolates in the present study. S: Streptomycin, CN: Gentamicin, K: Kanamycin, AMC: Amoxicillin-clavulanic acid, CRO: Ceftriaxone, RF: Rifamycin, DO: Doxycycline, C: Chloramphenicol, SXT: Sulfamethoxazole-trimethoprim, E: Erythromycin, CIP: Ciprofloxacin and IPM: Imipenem.

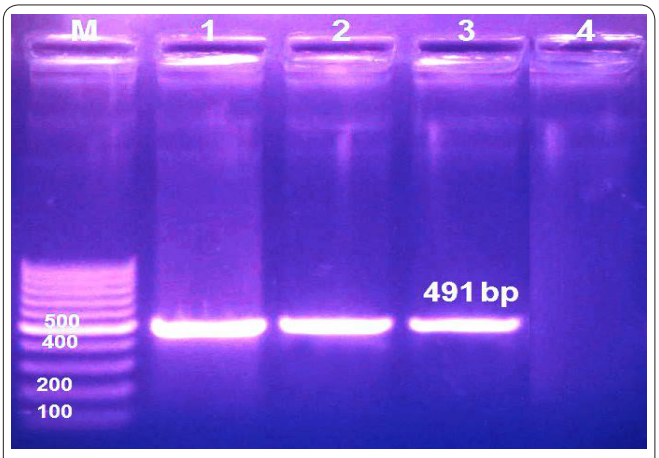

Figure 2. PCR amplification products with primers targeted against the integron. Lane M, 100-bp ladder; lanes 1-3, integron positive isolates**.** lanes 4, integron negative isolate.

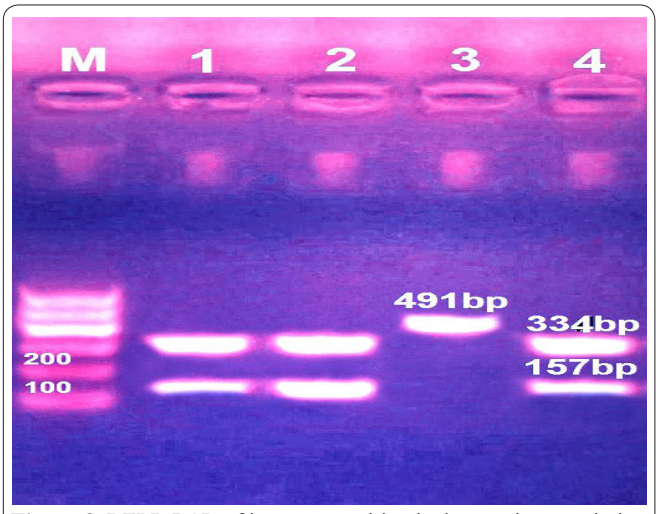

Figure 3. RFLP-PCR of integron positive isolates using restriction enzyme digestion. Lane M, 100-bp ladder; Lane 1, 2 and 4 (334 bp and 157 bp) represent integron class2. Lane 3(491bp) represents integron class1.

Antibiotic susceptibility

Among 110 tested Enterobacteriaceae isolates, absolute sensitivity was detected to imipenem (100%) but drug resistance was mostly observed for rifamycin (82.7%), erythromycin (67.2%) and amoxicillin-clavulanic acid (63%) (Figure1). Moreover, multidrug resistance (MDR) was observed in 48.18% (53/110) of the examined isolates and distribution of resistance among obtained six Enterobacteriaceae species, more than 50% of *E. coli*, *Salmonella* and *Klebsiella* spp were resistant to at least five different antimicrobial agents tested. On the contrary, Proteus isolates were the most susceptible species to all antimicrobial agents used in our study.

PCR screening for integrons

From examined 53 MDR Enterobacteriaceae isolates, integrons were detected in 38(71.6%) isolates (19 *Salmonella*, 12 *E. coli,* 3 *Proteus*, 3 *Klebsiella* and *1Citrobacter* species) Table (1). Class 2 integrons were more common than class 1 integrons. Of the 38 integron positive isolates, 3(5.66%) carried a single class 1 integron and 35(66.03%) carried class 2 integron Figure (2, 3). Moreover, no strain was found to contain both class 1 and 2 integrons. The amplification products of class 2 integrons varied in size between 2000, 1700, 900 and 650 bp, while class 1 integrons harbored amplification products 1600, 1000 and 800 bp Figure (4, 5). The association of resistance to antibiotics and integrons is shown in Table (3).

Table 1. Distribution of integron classes in different species of MDR Enterobacteriaceae isolates.

Bacterial species	Total	Integron positive (%)	Class1 integron (%)	Class2 integron (%)
S. Typhimurium	11	8 (72.7%)	1	7
S. Enteritidis	6	5(83.3%)		5
S. Virchow	2	2(100%)	1	1
S. Kentucky	2	2(100%)		2
S. Birkenhead	2	2(100%)	1	1
Escherichia O158	9	7(77.7%)		7
Escherichia O78	2	2(100%)		2
Escherichia O111	2	1(50%)		1
Escherichia O27	2	1(50%)		1
Escherichia O26	1	1(100%)		1
K. pneumonia	6	3(50%)		3
K. oxytoca	2	0(0%)		
C. freundii	2	1(50%)		1
Proteus mirabilis	2	2(100%)		2
Proteus vulgaris	1	1(100%)		1
E. hafanie	1	0(0%)		
Total	**53**	**38(71.6%)**	**3(5.66%)**	**35(66.03%)**

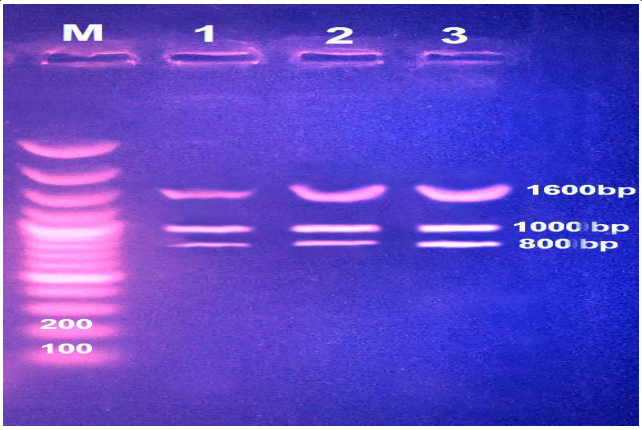

Figure 4. PCR amplification of class1 integron-variable regions generated with hep58, hep59 primers Lane M, 100-bp ladder; lanes 1-3, amplicons from *Salmonella* isolates (amplicon sizes in parentheses); *dfrA12-orfF-aadA27*(1600 bp), *aadA23* (1000 bp), *dfrA15* (800 bp)

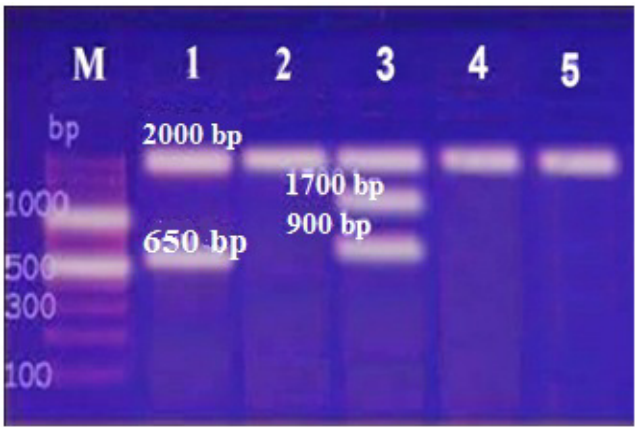

Figure 5. PCR amplification of class 2 integron-variable regions generated with hep51, hep74 primers. Lane M, 100-bp ladder; lanes 1: amplicons from *Salmonella* isolates (amplicon sizes in parentheses); *dfrA1-sat2- aadA30* (2000 bp), *sat*2 (650 bp). Lanes 3: amplicons from *Salmonella* isolates, *sat2-aadA1 (*1700 bp), *catB2* (900, bp)**.** Lanes 4: amplicons from *Salmonella* isolates, (2000 bp) & (900 bp)**.** Lanes 2 &5: amplicons from *Klebsiella* isolates (2000 bp).

Characterization of integron gene cassettes

The integron gene cassettes regions were cloned and sequenced. Analysis detected the following gene cassettes within class 1 integrons: [*dfrA12- orfF –aadA27* (1.6 kbp), *aadA23* (1.0 kbp) and *dfrA15* (0.8 kbp)] all present within 3 MDR isolates harbored integron class1. Class 2 integron present in 35 MDR isolates which carried genes cassette arrays: *dfr*A1-*sa*t2-*aad*A30 (2 kbp) in all isolates, *sat*2-*aad*A1 (1.7 kbp) in only one isolate, *catB2* (0.9 Kbp) in 4isolates and *sat2* (0.650 kbp) in 3 isolates. All gene cassettes region sequences deposited into GeneBank with accession numbers Tables (2). The most prevalence identified gene cassettes were: aminoglycoside adenylyltransferase *aadA (aadA1, 23, 27, 30*) conferring resistance to aminoglycosides: streptomycin and spectinomycin, trimethoprim reductase *dfrA (dfrA1*, *12, 15*) conferring resistance to trimethoprim, streptothricin acetyltransferase *sat2* resistance to streptothricin and *catB2* gene conferring resistance chloramphenicol. Interestingly, *sat2* gene cassette has a single amino acid mutation at a.a no. 2 (K (Lys) AAG: T (Thr) ACG) and one silent mutation (ATT: ATC (I Ile)).

Discussion

Antimicrobial resistance has become a serious public health threat throughout the world as therapeutic options for several infectious diseases are currently limited by the presence of multidrug-resistant bacteria. Therefore, surveillance for antimicrobial susceptibility in Enterobacteriaceae is imperative because species of this family are among the most significant and prevalent avian pathogens which can be transmitted to humans through the food chain (13).

In this context, the characterization of antimicrobial resistance of Enterobacteriaceae isolates obtained from broiler chicken may help in understanding the role of practices, supplies, equipment, the external and internal environments in the re-entry and maintenance of resistant isolates in poultry farms. Our study, therefore, was designed to investigate the antimicrobial resistance rates and genetic determinants of MDR Enterobacteriaceae isolates from different poultry farms in Sharkia governorates.

Findings from the present study show six genera of Enterobacteriaceae members were obtained with high isolation rate. The genera were *Salmonella* (45%), *E. coli* (40%), *Proteus* (10%), *Klebsiella* (8%), *Citrobacter* (4%) and *Enterobacter* species (3%). These results are in agreement with the previous reports of (14, 15; 16) that isolated these genera from several farms but with different percentages. This variation in the prevalence rates can be output for wide differences in the scheme for sampling, the types of samples, Enterobacteriaceae detection protocol, and geographical location.

Serotyping results revealed that *S. Typhimurium* and *Escherichia* O158 were the predominant serotypes as previously mentioned by (17) and (18).

Resistance development is possibly associated with the long-term and widespread use of antimicrobials. In the present study, the antimicrobials used against the isolates produced varying reactions. The best overall

Table 2. Accession No. of gene cassettes sequences deposited into GeneBank.

Isolates	Strain	Gene cassettes	Accession No.
S. Birkenhead	MAS2016	*dfrA15*	KY009928
S. Typhimurium	MAS2/Egy/16	*dfrA12-orfF-aadA27*	KY064172
S. Virchow	MAS3/Egy/16	*aadA23*	KY098819
Proteus vulgaris	MAS1/Egy/16	*catB2*	KY026608
Proteus vulgaris	MAS1/Egy/16	*sat2*	KY026607
Escherichia O158	MAS5/Egy/16	*dfrA1-sat2- aadA30*	KY070366
S. Typhimurium	MAS4/Egy/16	*sat2- aadA1*	KY050781

Table 3. Association between antibiotic resistance pattern and integron positive Enterobacteriaceae isolates.

No.	Serotype	Resistance Profile	Integron class	Integron amplicon size (bp)	Gene cassettes
1	*S. Birkenhead*	CRO,CN,RF,C,SXT,AMC,DO,S,E	*Int 1*	1600, 1000 , 800	*dfrA12-orfF-aadA27, aadA23, dfrA15*
2	*S. Virchow*	CN,RF,C,SXT,AMC,DO,S,E	*Int 1*	1600, 1000 , 800	*dfrA12-orfF-aadA27, aadA23, dfrA15*
3	*S. Typhimurium*	RF,SXT,C,AMC,S,E	*Int 1*	1600, 1000 , 800	*dfrA12-orfF-aadA27, aadA23, dfrA15*
4	*S. Typhimurium*	CN,CRO,RF,C,SXT,AMC,DO,S,K,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
5	*S. Typhimurium*	RF,C, AMC,E ,SXT	*Int 2*	2000, 900	*dfrA1-sat2- aadA30, catB2*
6	*S. Typhimurium*	RF,C,SXT,AMC,DO,S,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
7	*S. Typhimurium*	CRO, RF,AMC,DO,S,E,SXT	*Int 2*	2000	*dfrA1-sat2- aadA30*
8	*S. Typhimurium*	CRO,RF,C,AMC ,E, SXT	*Int 2*	2000, 1700, 900	*dfrA1-sat2- aadA30, sat2-aadA1, catB2*
9	*S. Typhimurium*	RF,C,AMC,DO,S,E,SXT	*Int 2*	2000	*dfrA1-sat2- aadA30*
10	*S. Typhimurium*	CIP,CN,RF,C,SXT,AMC,DO,S,K,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
11	*S. Enteritidis*	CIP,CN,RF,C,SXT,AMC,DO,CRO,K,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
12	*S. Enteritidis*	CIP,CN,RF,C,SXT,AMC,DO,S,K,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
13	*S. Enteritidis*	RF,C,AMC,S,E,SXT	*Int 2*	2000	*dfrA1-sat2- aadA30*
14	*S. Enteritidis*	CRO,RF,C,AMC,DO,S,SXT	*Int 2*	2000	*dfrA1-sat2- aadA30*
15	*S. Enteritidis*	RF,CRO,AMC,SXT,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
16	*S. Virchow*	CIP,CRO,RF,C,SXT,AMC,DO,S,K,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
17	*S. Birkenhead*	CRO,RF,C,AMC,S,K,E, SXT	*Int 2*	2000, 650	*dfrA1-sat2- aadA30, sat2*
18	*S. Kentucky*	CIP,CRO,RF,C,SXT,AMC,DO,S,K,E	*Int 2*	2000, 650	*dfrA1-sat2- aadA30 , sat2*
19	*S. Kentucky*	CRO,RF,C,SXT,AMC,DO,S ,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
20	*Escherichia O158*	SXT,CN,K,AMC,S,CRO,DO,RF,C, CIP,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
21	*Escherichia O27*	SXT,CN,K,AMC,S,CRO,DO,RF,C, CIP,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
22	*Escherichia O111*	SXT,CN,K,AMC,S,CRO,DO,CIP,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
23	*Escherichia O158*	SXT,CN,K,AMC,S,CRO,DO,RF,C, CIP,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
24	*Escherichia O26*	SXT,CN,AMC,RF,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
25	*Escherichia O26*	SXT,K,AMC,S,CRO,RF,C,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
26	*Escherichia O78*	SXT,CN,K,AMC,S,CRO,DO,RF,CIP,E,C	*Int 2*	2000, 900	*dfrA1-sat2- aadA30, catB2*
27	*Escherichia O27*	SXT,K,AMC,S,CRO,RF,C,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
28	*Escherichia O26*	CN,AMC,S,CRO,CIP,E, SXT	*Int 2*	2000	*dfrA1-sat2- aadA30*
29	*Escherichia O158*	SXT,CN,K,AMC,S,CRO,DO,RF,C, CIP,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
30	*Escherichia O111*	SXT,K,AMC,S,RF,C,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
31	*Escherichia O158*	SXT,DO,AMC,S,RF,C,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
32	*K.pneumoniae*	SXT,CN,K,AMC,CRO,RF,C,CIP,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
33	*K.pneumoniae*	SXT,K,DO,RF,CN,S,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
34	*K.pneumoniae*	SXT,CN,K,AMC,S,CRO,DO,RF,C, CIP,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
35	*P. mirabilis*	SXT ,K,CRO,AMC,S ,RF,C,CN,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
36	*P. mirabilis*	SXT ,AMC,S ,RF,C,E	*Int 2*	2000	*dfrA1-sat2- aadA30*
37	*P. vulgaris*	S,AMC,DO,RF,C,E,SXT	*Int 2*	2000, 900, 650	*dfrA1-sat2- aadA30 , catB2 ,sat2*
38	*C. freundii*	SXT,CN,K,AMC,C,RF,S,E	*Int 2*	2000	*dfrA1-sat2- aadA30*

potency was seen with ciprofloxacin and imipenem. However, high resistance rates noticed with *Klebsiella* spp. against ciprofloxacin (75%) are in accordance with the resistance rates observed by (14) and (19). In addition, high frequencies of resistance were found to rifamycin (82.7%), erythromycin (67.2%) and amoxicillin-clavulanic acid (63%) as reported previously by (20, 21). Furthermore, 48.18% of isolates tested exhibited multidrug resistance, which was higher than that recorded in Japan and Nigeria by percentage 40.6% and 32.1%, respectively (22,16) and lower than that obtained in China by percentage 85% (23). The consequence of this behavioral change over the years and countries is the gradual emergence of resistant bacterial strains refractory to multiple antimicrobial agents (24, 25).

The integron acquisition is considered the major cause of multiple resistances of enteric bacteria. A number of studies investigated the occurrence of integrons in selected populations of avian isolates from gram-negative bacilli with wide prevalence range especially class 1 and 2 integrons (26; 27, 28).

In this study, we found that a large percentage (71.6%) of MDR Enterobacteriaceae isolates harbored integrons and this prevalence was higher than those re-

corded by (29) in Egypt (59.3%), (30) in Korea (51.1%), (31) in the United States (52%). This could be attributed to the development of antibiotic resistance and diverse geographical distribution. We also found the highest percentage of integrons in *Salmonella* (50%) which correlate with the reports of (32) and (33).

Our data found that prevalence of class 1, 2 were 5.66% and 66.03%, respectively revealed that class 2 integron represented surprisingly high incidence among MDR isolates closer to the findings of (34, 35) and opposed to most previous studies which proved the highest frequencies of occurrence of class 1 integrons in poultry isolates than class 2 (36, 37). The gene cassettes ranged between 800 base pairs and 1600 base pairs in class 1 integrons, and 650 base pairs to 2000 base pairs harbored in class 2 integrons. Many researchers published similar results confirming our investigation (38; 39, 33).

Sequence analysis revealed that class 1 integrons were detected only on three *Salmonella* isolates which carried the same three cassettes (*aad*A23, *dfr*A15, and *dfr*A12-*orf*F-*aad*A27). These cassettes had been described in many other investigations as well, indicating a wide distribution of class 1 integrons containing these cassettes which conferring aminoglycoside and trimethoprim resistance (40, 41, 27). The wide distribution of this resistance is likely to be due to the selection pressure and high use of trimethoprim and aminoglycosides in farms in our region which was also evidenced elsewhere (42).

Furthermore, four distinct cassettes were completely determined among class 2 integrons positive isolates. The first comprised already known *dfrA1, sat2,* and *aadA30*, which confers resistance to trimethoprim, streptothricin, and the aminoglycoside, respectively. This array was detected most frequently in class 2 integrons and called classic cassette (11, 43). Moreover, the second one was identified as *sat2-aadA1* which mediates resistance to streptothricin and aminoglycoside in one strain of *S. enterica* serovar Typhimurium with the knowledge that streptothricin antibiotics have not been used as veterinary therapeutics, but have been used as growth promoters since the mid-1980s in the former German Democratic Republic (44). This array was previously detected in *Citrobacter freundii* isolate from cattle faeces in Australia at the same amplicon size (45).

The third type of class 2 integron amplicon was the *cat*B2 gene cassette (100 % identical to *ca*tB2 in GQ422827 from a class 1 integron) which may be constituted through the eradication of *catB2* from a class 1 integron and inserted into the most common class 2 integron array conferring resistance to chloramphenicol as previously stimulated by (46). The last cassette harbored a novel variant of a *Sat*2 cassette. The 133 amino acid *sat 2* protein described in the databases under accession No. (KY026607) as streptothricin acetyltransferase differs by a single amino acid substitution at position 2 from the so-far knew *sat2* proteins: *Lys* in the present case versus *Thr* in all other s*at2* sequences. This suggests that gene cassettes are becoming more diverse and evolutionarily diverging from the previous ancestors.

Additionally, all the isolates carrying integrons were also resistant to sulfamethoxazole-trimethoprim; these results are consistent with results of previous studies investigating an association between the presence of integrons and the antimicrobial reaction of enteric bacteria to trimethoprim and sulfa compounds (47, 48).

As mentioned before, the phenotypic resistance to a specific antibiotic was observed in most of the isolates carrying the corresponding gene cassette which was in agreement with (49; 50, 51) Nevertheless, 28.30% of integron-negative strains were multidrug resistant. This result indicates that the presence of integrons represents only one among many factors influencing the development of multidrug resistance. Several other factors promoting the emergence, selection and dissemination of resistant enteric strains as antibiotic usage, incomplete therapy, use of nontherapeutic antimicrobial growth promotants as feed additives for poultry (52), inappropriate use of disinfectants in farm environments (53), and mutations in the *mar* (multiple- antibiotic resistant) locus regulation (54).

In conclusion, the present study gives a representative picture of high integrons prevalence in isolates of enteric bacteria obtained from broiler chickens, and their significant association with reduced susceptibility to a range of antibiotics. Thus, it is crucial to track the evolution of multidrug-resistant isolates from poultry which may be fundamental to estimate the health risk and prevent the spread of particular antibiotic resistance determinants to humans.

Interest conflict

The authors manifested that they have no conflicts of interest.

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