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Association of Antiseptic Resistance Gene (*qacEΔ1*) with Class 1 Integrons in *Salmonella* Isolated from Broiler Chickens

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ABSTRACT

Salmonella enterica is considered a zoonotic pathogen that acquires antibiotic resistance in livestock. In the current study, a total of 18 *Salmonella enterica* isolates recovered from cloacal swabs of diseased and freshly dead broilers were serotyped and assessed for susceptibility to clinically important antibiotics. The multi-resistant isolates were examined for the presence of the antiseptic resistance genes including quaternary ammonium (*qacEΔ1)* and class 1 integron-integrase (*intI1*) by PCR. The results of serotyping of 18 *Salmonella* isolates indicated that five isolates belonged to *Salmonella* Typhimurium, four isolates belonged to each of *Salmonella* Kentucky and *Salmonella* Enteritidis, three isolates belonged to *Salmonella* Molade and one isolate belonged to each of *Salmonella* Inganda and *Salmonella* Larochelle. Fifteen *Salmonella* isolates (83.3%) were multi-resistant to at least three antibiotics with a multidrug resistance index value of 0.473. All of the *intI1*-positive strains carried *qacEΔ1*, confirming that the *qacEΔ1* gene is linked to the integrons. The study concluded that the presence of the *qacEΔ1* resistance gene and class 1 integrase in multi-drug resistant *Salmonella* strains might be contributed to co-resistance or cross-resistance mechanisms.

Key words: *intI1,* Multidrug-resistant *Salmonella,* PCR, *qacEΔ1*

INTRODUCTION

Salmonella Typhimurium continues to be among the most common serovars isolated from poultry and a common cause of human salmonellosis (Foley et al., 2011). Salmonellae are prevalent in the environment and are found in both domestic and wild animals as pathogens or commensals. These bacteria can infect humans mainly via contaminated food such as meat, dairy products, eggs, fruits, vegetables (Yan et al., 2010).

The growing resistance of pathogenic bacteria to antimicrobials has raised the concern that the widespread use of antimicrobials in animal production may promote the development of resistant bacteria or resistance genes that can be transferred to bacteria which cause disease in humans (Wegener et al., 1997). Microbial resistance is the loss of sensitivity of a microorganism to an antimicrobial that it was originally susceptible. This resistance can be acquired by mutations in chromosomal DNA or the acquisition of extra-chromosomal genetic materials through plasmids and transposons (Vázquez et al., 2002). Zhang et al. (2004) studied 33 isolates of *Salmonella* among healthy people in China and found that all isolates were susceptible to ceftriaxone and 11 isolates harbored class 1 integron. It has been also stated that different serotypes of the genus *Salmonella* are resistant to various antimicrobials and carry class 1 integron, which is involved in antimicrobial multi-resistance (Vazquez et al., 2005). In addition, the strains harboring integrons exhibit the strongest resistance patterns (Muñoz et al., 2000).

González et al. (1998) published the first evidence of the presence of integrons in Gram-negative bacilli isolated from biological residues in Chilean hospitals and found the integrons are commonly associated with the family Enterobacteriaceae. Integrons function as a system that captures genes that confer selective advantages to the bacterium. Integrons allow the bacterium to rapidly adapt to ecological changes, due to their capacity to recognize a wide variety of recombination sequences, their exchange capacity and remote origin (González et al., 2004). Integrons are genetic elements in plasmids and transposons and frequently contain one or more genes encoding resistance to antimicrobials (Stokes and Hall, 1989). Four classes of integrons are known (1, 2, 3, and 4), with class 1 being predominant among the members of this family both in the normal and pathogenic microbiota of

animals (Goldstein et al., 2001). Integrons contribute to the spread of antimicrobial resistance by gene transfer in a variety of enteric bacteria, including *Salmonella* (Maynard et al., 2003).

Disinfectants are, however, employed during production breaks as a routine part of the management of poultry farms. Disinfectants such as Quaternary Ammonium Compounds (QACs) that have been introduced into farm environments. A particular concern is that repeated usage of disinfectants may give rise to the selection and persistence of bacteria with reduced susceptibility not only to the antiseptics but possibly to antibiotics as well (Randall et al., 2004). QAC gene which is responsible for resistance to quaternary ammonium compounds and disinfectants located on the 3' regions of class 1 integron (Mazel, 2006). The mutant type QAC gene recorded high prevalence among *Salmonella* Typhi isolates (Hindi et al., 2014).

Chuanchuen et al. (2007) recorded that all of the *intI1*-positive strains carry *qacEΔ1*in 3′ conserved segment, confirming that the *qacEΔ1*gene is linked to the integrons. QAC resistance and dissemination are very important in the context of the global antibiotic resistance problem, also exposure to QACs results in the dissemination of integrons (Gillings, 2014). There is a link between antibiotic resistance in nature and clinical settings, which is favored by exposure to QACs (Forsberg et al., 2012).

The present study aimed to detect class 1integron (*intI1*) gene associated with antiseptic resistance gene (*qacEΔ1*) in *Salmonella* serotypes, and correlate the presence of these genes with multi-resistance to antimicrobials, as verified by the plate inhibition test.

MATERIALS AND METHODS

Ethical approval

The research protocol was reviewed and approved by the Institutional Animal Care and use Committee (VetCU02122019103).

Sampling

Cloacal swaps were collected aseptically from 100 chickens suffering from digestive, respiratory and/or locomotor disorders. The samples were then transported in 1.5 mL tubes containing 750µL of Brain Heart Infusion (BHI) broth refrigerated in the icebox to the Laboratory of Poultry Diseases Department, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt.

Isolation and biochemical identification of *Salmonella* spp.

For isolation of *Salmonella* spp., the following method was used in brief: inoculated BHI broth tubes were incubated at 37^{o}C for 18 hours then a loopful was transferred to Rappaport-Vassiliadis broth then incubated at 41^{o}C aerobically for 24 hours. Samples were streaked onto Brilliant Green agar with Novobiocin (40 µg/mL) and inoculated *Salmonella-Shigella* agar and incubated for 24 hours at 37^{o}C aerobically. The isolated pure cultures of *Salmonella* spp. were biochemically identified using the following tests; oxidase, indole, methyl red, Voges Proskauer, citrate utilization, urea hydrolysis, triple sugar iron agar and lysine decarboxylase (Quinn et al., 2002).

Serological identification

Isolates with biochemical profile compatible with *Salmonella* spp. were identified serologically using antisera (DENKA SEIKEN Co., Japan) in agglutination tests on the basis of somatic O antigen and phase 1 and phase 2 flagella antigens according to the Kauffmann-White scheme (Grimont and Weill, 2007).

Antibiotic susceptibility

All *Salmonella* serotype isolates were studied via the disk diffusion method to evaluate their resistance to antibiotic disks. The criteria proposed by the National Committee for Clinical Laboratory Standards (CLSI, 2013) was used to determine susceptibility rates. The following 13 antibiotic discs (Oxoid) used in the current study were: erythromycin (15µg), amoxicillin (30 µg), cephradine (30 µg), colistin (10 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), cefoxitin (30 µg), gentamicin (10 µg), penicillin (10 µ), neomycin (10 µg), streptomycin (10µg), florfenicol (15 µg) and amikacin (15 µg).

Multidrug resistance index

Resistance to more than three antibiotics was recorded as Multi-Drug Resistance (MDR). The MDR index of individual isolates was calculated by using the equation adopted by Chandran et al. (2008). In this equation, the number of antibiotics that the isolate was resistant to these was divided by the total number of antibiotics exposed. Isolates with MDR index values more than 0.2 or 20% were considered highly resistant.

$$\text{MDR index} = \frac{\text{Number of antibiotics resisted}}{\text{Total number of antibiotics used}} \times 100$$

Experimental Design

Suspension of *Salmonella* isolates, in a saline solution, was prepared with a 24h agar culture using the McFarland scale, a concentration of bacteria was established, it means that the suspension contained $1800x10^6$ *Salmonella* bacteria in 2 ml (Balicka et al., 2007). A volume of 2 ml of this suspension was administered to each of 30 six-day-old chicks. On the 15^{th} day, birds were humanely killed and both ceca and cecal tonsils were aseptically collected and cultured for the presence of *Salmonella* spp.

PCR amplification and DNA sequencing

Ten *Salmonella* isolates were tested for the presence of *qacEΔ1* and integrase gene (*intI1*) using PCR as the following:

DNA extraction

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted in 100 µl of elution buffer that was provided in the kit.

Oligonucleotide primer

Used primers were supplied by Metabion (Germany) and listed in table 1.

PCR amplification

Primers were utilized in a 25- µl reaction containing 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR products

The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products were loaded in each gel slot. GeneRuler 100 base pair DNA ladder (Fermentas, Sigma) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

RESULTS

The obtained results in the current study showed that on examination of 100 cloacal broiler chicken samples aseptically collected from diseased and freshly dead chickens, 18 *Salmonella* isolates were recovered with an overall percentage of (18%)**.** *Salmonella* isolates were motile, and they were positive with methyl red, citrate utilization, H2S, LDC, Arginine dihydrolase and xylose. However, they were negative with indole, Voges Proskauer, urease, Gelatin liquefaction, ONPG.

Serotyping of *Salmonella* isolates revealed that *Salmonella* Typhimurium was the most common serovar (5 isolates) followed by *Salmonella* Kentucky and *Salmonella* Enteritidis (4 isolates) and *Salmonella* Molade (3 isolates)**,** while *Salmonella* Larochelle and *Salmonella* Inganda were represented by one isolate for each of them (Table 2). The experimental chickens were infected with a suspension containing $1800x10^6$ bacteria in 2 ml. 85% of birds had intensive clinical symptoms, Ruffled feathers, diarrhea, weakness, and apathy. Postmortem examination revealed severe congestion in the intestines, swollen liver with necrosis and dehydration. Two cases died and *Salmonella* was re-isolated from the intestines and cecum.

The antibiotic resistance pattern of the 18 *Salmonella* isolates is shown in table 3. The obtained results showed that 100% of the isolates were susceptible to amikacin (100%) followed by Ciprofloxacin (88.89%) and gentamicin (72.3%), norfloxacin/florfenicol (66.7%) and streptomycin (61. 2%). High resistance rates were observed against penicillin (100%), followed by Amoxicillin (94.5%) and Erythromycin (83.3%). In addition, 15 *Salmonella* isolates (83.3%) were multi-resistant to at least three antibiotics with MDR index value of 0.473 of which 10 isolates were tested for *intI1* and *qacEΔ1* genes.

The class 1 integron was detected in 10 multidrug-resistant isolates giving characteristic bands at 280 base pairs (Figure 1). The *qacEΔ1* was also detected among DNA products of 10 multidrug-resistant *Salmonella* isolates giving characteristic bands at 362 base pairs (Figure 2).

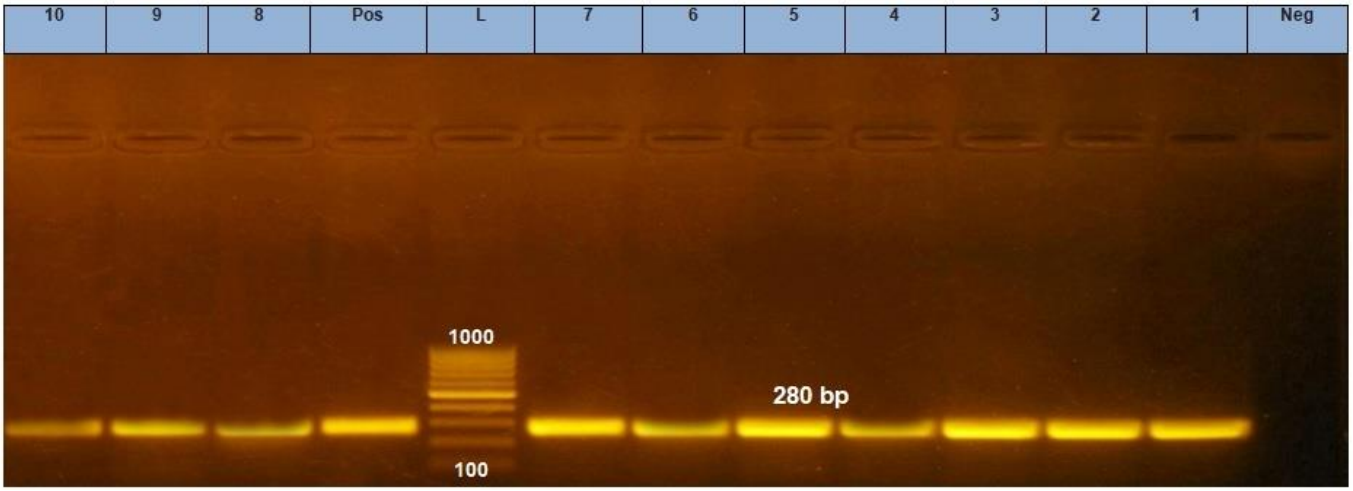

Figure 1. Agarose gel electrophoresis showing PCR amplification at 280 base pair fragment for class 1 integron (conserved segment) among DNA products of 10 multidrug-resistant *Salmonella* isolates collected from cloacal swaps from chickens, Egypt. L: 100 base pair DNA ladder, Neg: Negative Control, Pos: Positive control

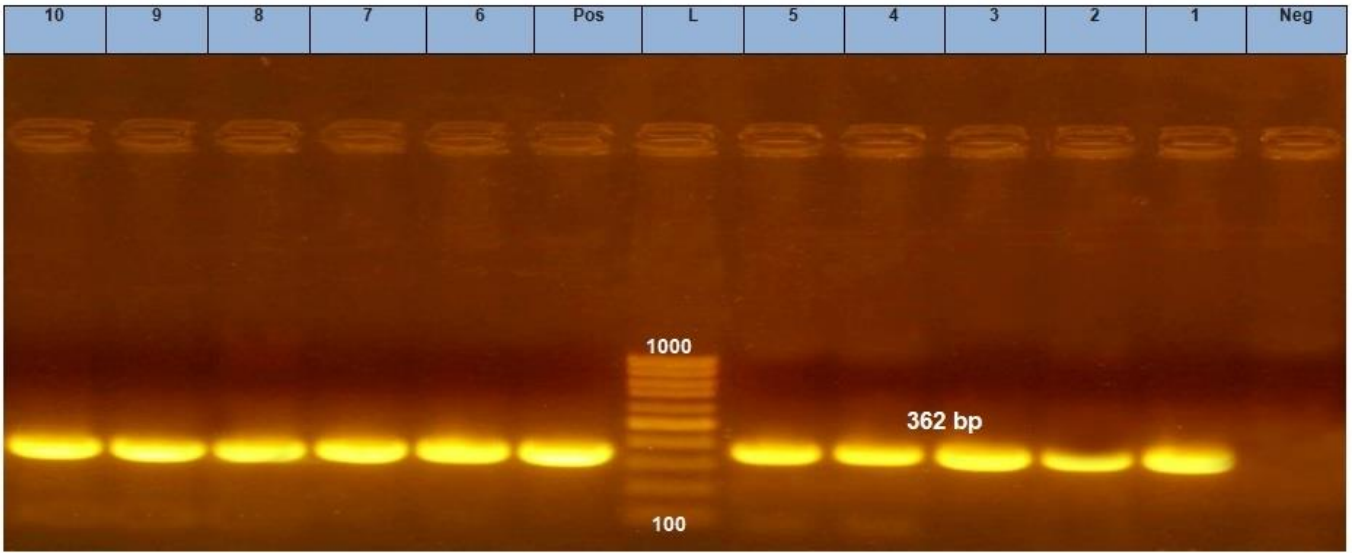

Figure 2. Agarose gel electrophoresis showing PCR amplification at 362 base pair fragment for *qacEΔ1* gene among DNA products of 10 multidrug-resistant *Salmonella* isolates collected from cloacal swaps from chickens, Egypt. L: 100 base pair DNA ladder, Neg: Negative Control, Pos: Positive control

Table 1. Primers sequences, target genes, amplicon sizes, and PCR cycling conditions

Target gene	Primers sequences (5'- 3')	Amplified segment (base pair)	Amplification (35 cycles)				Final extension	Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension		
qacEΔ1	F:TAAGCCCTACACAAATTGGGA GAT AT R:GCCTCCGCAGCGACTTCCACG	362	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Chuanchuen et al. (2007)
intI1	F:CCTCCCGCACGATGATC R:TCCACGCATCGTCAGGC	280	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Kashif et al. (2013)

R: reverse, F: forward

Table 2. Results from Serotyping of *Salmonella* isolates collected from cloacal swaps from chickens, Egypt

Strain	Prevalence	
	Number	%
Salmonella Molade	3	16.6%
Salmonella Enteritidis	4	22.2%
Salmonella Kentucky	4	22.2%
Salmonella Inganda	1	5.5%
Salmonella Typhimurium	5	27.7%
Salmonella Larochelle	1	5.5%

Table 3. Antibiotic resistance pattern of *Salmonella* isolates collected from cloacal swaps from chickens, Egypt

Antibiotics discs	*Salmonella* isolates (total number:18)			
	Resistant		Sensitive	
	%	Number	%	Number
Amikacin (15µg)	0	0	100	18
Amoxicillin (25µg)	94.5%	17	5.5%	1
Colistin (30µg)	22.2%	4	77.8%	14
Cephradine (30µg)	55.5%	10	44.5%	8
Ciprofloxacin (5µg)	11.11%	2	88.89%	16
Cefoxitin (30µg)	66.6%	12	33.4%	6
Erythromycin (15µg)	83.3%	15	16.7%	3
Florfenicol (15µg)	33.3%	6	66.7%	12
Gentamicin (10µg)	27.7%	5	72.3%	13
Norfloxacin (10µg)	33.3 %	6	66.7%	12
Neomycin (30µg)	50%	9	50%	9
Penicillin (10u)	100%	18	0%	0
Streptomycin (10µg)	38.8%	7	61.2%	11

DISCUSSION

The obtained results in the current study showed that on examination of 100 chicken cloacal swabs samples aseptically collected from diseased and freshly dead chickens, 18 *Salmonella* isolates were obtained with a percentage of 18%. However, previous studies reported slightly lower values for *Salmonella* isolation. In this respect, the prevalence of *Salmonella* was 12.8% in broilers farms in Egypt (Orady et al., 2017), 12.6% in poultry farms in Kuwait (Al-Zenki et al., 2007) and 10% were isolated from internal organs (liver, spleen, and heart) of broilers (El-Azzouny, 2014). However, a much lower prevalence of *Salmonella* was reported in other localities in Egypt where an overall prevalence of 1.7% (Ahmed et al., 2009), 2% and 2.5% (Mohamed et al., 1999) was found in Sharkia, Gharbia, and Kafr-Elsheikh governorates, respectively. Also, other studies showed more variable prevalence rates of *Salmonella* isolates worldwide. *Salmonella* isolates were found in 3.1% of internal organs of chickens in North Vietnam (Hanh et al., 2006), but Molla et al. (2003) isolated *Salmonella* from 34.5% of chicken samples in Ethiopia. The above-mentioned discrepancy in prevalence rate of *Salmonella* spp. could be attributed to the disparity in sampling schemes, types of samples, protocols of *Salmonella* detection and geographic differences as well as hygienic practices.

In concordance with the previous study by Bywater et al. (2004), the isolation of *Salmonella* with a higher percentage from broiler chickens necessitate the application of biosecurity program inside farms beside using alternatives to antimicrobials such as bacteriophages and herbal extracts for cutting the horizontal transmission of *Salmonella* to broiler carcasses (Elkenany et al., 2019).

In agreement with previous studies, *Salmonella* Typhimurium was the most common serovar isolated from broilers in many countries (Verma and Gupta, 1995; Moussa et al., 2010; Rabie et al., 2012; Borges et al., 2015; Ammar et al., 2016; Orady et al., 2017). It accounted for 27. 7% of total *Salmonella* isolates in the current study. Other serotypes isolated in the present study were *Salmonella* Enteritidis and *Salmonella* Kentucky with a percentage of 22.2% and *Salmonella* Molade with a

percentage of 16.6%, while *Salmonella* Inganda *and Salmonella* Larochelle recorded the lowest percentage (5.5%). These results are consistent with the results of Orady et al., (2017) who mentioned *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most common serovars recording 15.6%, while *Salmonella* Kentucky and *Salmonella* Molade accounted for 6.2% and 3.1%, respectively.

Regarding the sensitivity pattern of each of the 18 isolated *Salmonella* serovar, 15 *Salmonella* isolates had multi-resistance to at least three antibiotics with an MDR index value of 0.473, whereas 3/18 (16.7%) had MDR index value of 0.112 ≤0.2. These results differ from those reported by Orady et al. (2017) who mentioned that 62.5% of *salmonella* isolated from chickens showed MDR phenotypes to at least three classes of antimicrobials. Also, Singh et al., (2010) reported that all tested *Salmonella* spp. isolates from chickens were resistant to at least one antimicrobial compound. This increased MDR could be attributed to the wide range, irresponsible and misuse of antibiotics in poultry farms.

In the present study, all isolates were fully susceptible to amikacin (100%), which was the most effective antibacterial agent against *Salmonella* infection followed by ciprofloxacin (88.89%), Colistin (77.8%), gentamicin (72.3%) followed by streptomycin (61.2%). Comparable findings have been reported by Orady et al. (2017) and Łukasz and Popowska (2016).

It has been stated that there is an association between class 1 integrons and the development of antibiotic resistance (Guerra et al., 2003; Orady et al., 2017). In addition, class 1 integrons are the most frequently found integrons that contribute to MDR in gram-negative bacteria (Fluit and Schmitz, 2004; Hsu et al, 2006). In the current study, class 1 integron was screened among the obtained multidrug-resistant *Salmonella* isolates. PCR amplification revealed that Class 1 integrons were detected in 10 tested MDR *Salmonella* isolates (100 %). In agreement with Ammar et al. (2016), class 1 integrons contribute significantly to antibiotic resistance in *Salmonella* isolates. There is a discrepancy in the percentage of *Salmonella* isolates expressing the presence of class 1 integrons as revealed by previous studies. Comparable results to the current results have been obtained by Antunes et al. (2004) and Orady et al. (2017) who mentioned that class 1 integrons were detected in almost all isolates (99% and 95%, respectively). However, lower percentages have been demonstrated by Gautam et al. (2017) in India (69.9%) and Shahada et al. (2006) in China (24.5%). Contrarily, Okamoto et al. (2009) and Hindi et al. (2014) recorded that class 1integron (*intI1) gene* was not observed in any of the 100 multidrug-resistant *Salmonella* spp. as it was not detected by PCR. The integron has also been found in other Enterobacteriaceae but it is not very frequent as in *Salmonella* (Guerra et al., 2004). The uncontrolled use of antibiotics would increase the number of multidrug-resistant isolates and integrons prevalence, which by time, could be a significant public health threat (Orady et al., 2017).

As demonstrated in the present study, all isolates expressing class 1 integrons were positive for the presence of the *qacEΔ1g*ene, indicating the positive correlation between them. In the same context, class 1 integrons were associated with *qacEΔ1* and *sul1* and commonly detected in clinical isolates of *Salmonella* (Hsu et al., 2006). In addition, Chuanchuen et al. (2007) mentioned that the *intI1* gene was identified in 23 isolates (70%) with *qacEΔ1* and all of the *intI1*-positive strains carried *qacEΔ1* in 3´ conserved segments, confirming that the *qacEΔ1* gene is linked to the integrons. Moreover, Gaze et al. (2005) reported a link between increased class 1 integron frequency as well as increased QAC resistance.

Recently, an unusual 3´ conserved sequence regions with QAC linked to a *sul3* domain was found in plasmid-borne class 1 integrons in different *Salmonella* serovars (Antunes et al., 2004). Also, the 5´ CS region contains *intI1*, the typical 3´ CS region usually consists of *qacEΔ1;* encoding resistance to quaternary ammonium compounds, *sul1;* encoding resistance to sulphonamide (Fluit and Schmitz, 2004). Integrons play a significant role in the acquisition and mobilization of QAC resistance genes (Cambray et al., 2010). Also, plasmid-associated QAC resistance genes are transferred between non-pathogenic and pathogenic bacteria exposed to QACs, a process that also leads to the co-selection of resistance to other contaminants (Katharios et al., 2012). So, Antibiotic and QAC resistance genes are both carried on class 1 integrons, which raises concerns that QAC exposure resistance may co-select for antibiotic resistance by selecting for class 1 integrons (Chuanchuen et al., 2007).

On the contrary, *Salmonella* Enterica strains positive for *qacE1* but without *intI1* were also identified. Carriage of the *qacE1* gene may be on other elements or integrated into the chromosome (Chuanchuen et al., 2007). Also, the class 1 integron gene (*qacE1-SulI*) was not detected in any *Salmonella* isolates (Diarrassouba et al., 2007).

CONCLUSION

The majority of *Salmonella* isolates were multi-drug resistant to at least three antibiotics. The presence of integrons among *Salmonella* isolates is considered to be an important contributor to the development of antibiotic resistance**.** The presence of class 1 integrons in all of the *qacEΔ1*-positive strains confirms a significant association between them and confers cross-resistance to different groups of antibacterial. Increasing resistance among *Salmonella* isolates harboring class 1 integron and *qacEΔ1g*ene are linked to the excessive use of antimicrobials and disinfectants in broilers farm.

DECLARATION

Competing interests

The authors declare no conflict of interest.

Authors' contributions

Both authors contributed equally to this work.

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