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Risk of *Staphylococcus aureus* Isolated from Poultry Meat of Chicken with Arthritis in Poultry Farms

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Abstract

Staphylococcus aureus is a major pathogen that affects both people and animals. *Staphylococcus aureus* causes food poisoning in addition to invasive diseases as arthritis and septicemia. This study was done on 70 chicken samples obtained from 7 different farms of chickens with symptoms of arthritis in Kafr El-sheikh government, Egypt. In this study out of 70 samples of chickens from different farms, 37 (52.8%) samples were recognized as coagulase-positive staphylococci (CoPS) and 33 (47.1%) were recognized as coagulase-negative staphylococci (CoNS). By using the microtitre plate method, seven out of 37 (18.9%) CoPS were positive for biofilm production with variable degrees. The pattern of antibacterial sensitivity of 7 *Staphylococcus aureus* isolates against 12 commercially available antibiotic discs showed 100 % resistance to oxytetracycline then Amoxicillin (71.43%), Erythromycin (57.14%), Norfloxacin (14.29%), Tetracycline (42.86), Sulphamethoxazole (42.86%), Gentamicin (42.86%), Ampicillin (42.86%), kanamycin (28.57), cephatotin (28.57), doxycycline (0%) and the least was observed with chloramphenicol (0%). seven of positive *S. aureus* isolates were introduced in order to identify the staphylococcal enterotoxin genes, *SEA*, *SEB*, *SEC*, *SED*, and *SEE* and integron by PCR test Which 4 out of 7 isolates (57.1 %) were positive for *SEB* and *SED* only while were other isolate were negative for all *SE* gene. Class 1 integron cassettes were detected in 6 isolates from 7 (85.7%) of tested isolates. In conclusion, this is the first study to report the detection and identification of enterotoxin and class 1 integron in *S. aureus* isolated from poultry meat of chicken that suffered from arthritis.

KEYWORDS
Staphylococcus aureus, Biofilm, Antibiotic sensitivity, Enterotoxins, Class 1 integron, PCR, Poultry, Arthritis.

INTRODUCTION

Syndromes of Staphylococcal Infectious Diseases of chickens are seen as a financial burden on the poultry industry globally and a danger to the economy. (Mkize *et al*., 2017). Specifically, *S. aureus* can give rise to severe economic losses in the poultry industry. This is due to lameness, mortality, decreased weight gain, decreased egg production, and condemnation of carcasses at the slaughterhouse (Andreasen, 2003; El-Tawab *et al*., 2017). A certain *S. aureus* strain's virulence is related to a confluence of extracellular components, toxins, and the strain's invasive and sticky characteristics as a result of adhesins, biofilm formation, and phagocytosis resistance (Gordon and Lowy, 2008).

Staphylococcus aureus has been known to cause foodborne illness in both people and animals since the 1880s. In 1930, it was discovered that staphylococcal food poisoning was caused by an exotoxin rather than conventional colonization and infection by the bacteria. The five most well studied enterotoxins are considered classical enterotoxins (*SEA*, *SEB*, *SEC*, *SED*, and *SEE*) encoded by specific enterotoxin genes denoted as the *sea* to *see* (da Silva *et al*., 2015).

Other than staphylococcal food poisoning, the etiology of *S. aureus* illness has also been linked to the staphylococcal enterotoxins. There is a significant correlation between invasive *S. aureus* infections in people and the enterotoxins *SEA*, *SEB*, *SEC*, and *SED* (Fisher *et al*., 2018). Most frequently, contaminated chicken and turkey flesh, joints, tendon sheaths, and bones are where *Staphylococcus aureus* is found. (Szafraniec *et al*., 2020).

Biofilm is composed of multilayered cell clusters imbedded in an extracellular polysaccharide matrix. (slime). This facilitates adherence and shields the microorganisms from host immune defenses and antimicrobial therapy (Limoli *et al*., 2015). Biofilm formation starts with bacterial adhesion to a surface, followed by accumulation, maturation, and detachment phases required for staphylococci dissemination (Foster, 2020). The inside of bacterial biofilms presents greater resistance to the opsonization by antibodies and phagocytosis (Hoyle and Costerton, 1991) which explains the chronic character of such infections. In addition, the synthesis of polysaccharide intercellular adhesin (PIA), which is regulated by the ica operon, is implicated in the development of multicellular biofilm (Le *et al*., 2018).

Integrons are genetic sets that have the ability to integrate mobile genetic components known as gene cassettes. Although class1 integrons are well established in playing a role in the dissemination of antibiotic resistance genes among Gram-negative bacteria, little is known about the prevalence of class 1 integrons in Gram positive bacteria, particularly *S. aureus* (Sabbagh *et al*., 2021). Class 1 integrons/cassettes may play an important role in the horizontal transfer of antimicrobial resistance genes between bacterial species from various sources or geographical areas (Li and Zhao, 2018).

The present study aimed to investigate antibiotic resistance,

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occurrence and distribution of integron cassette in multidrug-resistant *Staphylococcus aureus* isolates from Poultry Meat of Chicken suffered from arthritis in addition to detection of enterotoxin of isolated *S. aureus*.

MATERIALS AND METHODS

Sampling

A total of 70 samples of chickens that showed symptoms of arthritis were collected from 7 different farms all over Kafr Elsheikh governorate, Egypt from January 2020 to January 2022. Chickens were investigated for the presence of *Staphylococcus aureus* infection.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Care and Use of Laboratory Animals committee of Alexandria University. All methods were carried out in accordance with relevant guidelines and regulations (Ethics Committee, Faculty of Veterinary Medicine, Alexandria University, Egypt). The study was carried out in compliance with the ARRIVE guidelines.

Isolation and Identification of Staphylococcus aureus

A total of 70 chicken meat samples were inoculated into tryptic soya broth and incubated at 37°C for 18 h then sub cultured into nutrient agar for the detection of pigment production and Mannitol salt agar as a selective media for isolation and differentiation of *S. aureus* (Sharp and Searcy, 2006), semisolid nutrient agar medium was used for detection of bacterial motility and for preservation of bacterial culture, oxidation fermentation medium was used for identification and differentiation between genus micrococcus and *Staphylococcus* hydrogen peroxide (3%)was used for catalase test (Namvar *et al*., 2014), sterile solution of citrate sodium (4%) was used as anticoagulant for coagulase test in addition to citrate rabbit plasma (diluted 1:4), Gram's stain was used to stain bacterial isolate films for morphological characteristics and staining reactions. (Nawara *et al*., 2019). All media was prepared using distilled H_2O and autoclaved at 121˚C.

Antimicrobial sensitivity test

Antimicrobial susceptibility was tested by the single diffusion method according to Daka and Yihdego (2012) for *S. aureus*. Sensitivity discs with variable concentrations were used to determine the susceptibility of the isolated *S. aureus* strains (Oxoid Limited, Basingstoke, and Hampshire, UK). The antimicrobial sensitivity patterns of 7 *S. aureus* isolates were determined against 12 antimicrobial agents as in Table 3. Oxytetracycline (OT), Amoxicillin/Clavulinicacid (A/C), Erythromycin (E), Ampicillin (AM), Sulphamethoxazole/Trimethprim (SXT), Gentamicin(G), Tetracycline (TE), Kanamycin(K), Cephalotin (CN), Norfloxacin (NOR), Chloramphenicol (C), Doxycycline (DO). The inhibitory zone diameters were measured and interpreted according to Clinical and Laboratory Standards Institute (Wikler, 2006).

Detection of the formation of biofilms

To assess the amount of biofilm that *Staphylococcus* species produce, a tissue culture plate is used. by using a microtiter test (Daka and Yihdego, 2012). Trypticase soy broth is inoculated with an overnight culture of the isolate from nutrient agar plate (TSB). The main inoculums are then put into 96 wells of a flat-bottom microtiter plate and inoculated in TSB with 1% glucose at various dilutions (1:20, 1:40, 1:80, and 1:100). The wells are decanted and rinsed three times with phosphate buffer saline after the plates are covered and incubated at 37°C for 24 hours in an aerobic environment (PBS). Fix with methanol for 15 minutes after washing. The wells are then decanted and dyed for 20 minutes with crystal violet. Once again decanted, distilled water is used to clean the wells. The plate was then allowed to dry at ambient temperature for an hour, and the absorbance was then measured using a spectrophotometer at 620 nm OD in accordance with Deka (2014).

Molecular Identification of Staphylococcus aureus and Screening of Enterotoxin Genes and integron

The enterotoxin genes (*Sea*, *Seb*, *Sec*, *Sed*, and *See*) and the presence of integrons were tested in seven isolates of positive *S. aureus* broth culture using hep35&hep36 primers coding for integrase gene, and the gene cassettes within class 1 integrons were amplified using 5′-CS and 3′-CS primer pairs using PCR technique.

DNA extraction

DNA was extracted from samples according to the manufacturer's instructions for the QIAamp DNA Mini kit (Qiagen, Germany, GmbH). Table 1 lists the primers that were provided by Metabion (Germany).

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions of enterotoxin genes and integrase gene.

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Sea	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102	94˚C 5 min.	94˚C 30 sec.	57˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Mehrotra *et al*. (2000)
Seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164			57˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
Sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	451			57˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
Sed	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278			57˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
See	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209			57˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	
Integron *(hep 35 and hep 36 primers)*	TGCGGGTYAARGATBTKGATTT CARCACATGCGTRTARAT	491			55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	White *et al*. (2000)

RESULTS

Incidence of pathogenic Staphylococci in examined samples

As demonstrated in Table 2, out of 70 meat samples that were collected from chicken that suffered from arthritis, 37 samples were positive for pathogenic *Staphylococcus aureus* at a percentage of 52.8%.

Table 2. Percentage of positive samples for pathogenic *Staphylococcus aureus*.

Source of sample	Number of tested samples	Number of positive samples	%
Farm (1)	10	3	30%
Farm (2)	10	5	50%
Farm (3)	10	3	30%
Farm (4)	10	9	90%
Farm (5)	10	10	100%
Farm (6)	10	4	40%
Farm (7)	10	3	30%
Total	70	37	52.80%

Results of antibacterial sensitivity test

A total of 7 samples of positive *S. aureus* were introduced to antibiotic susceptibility test. After cultured in MSA and biochemical identification. The antimicrobial sensitivity pattern of *Staphylococcus aureus* against 12 commercially available antibiotic discs revealed 100% resistance to oxytetracycline, which was followed by amoxicillin (71.43%), erythromycin (57.14%), norfloxacin (14.29%), tetracycline (42.86), Sulphamethoxazole (42.86%), gentamicin (42.86%), ampicillin (42.86%), kanamycin (28.57), cephatotin (28.57), doxycycline (0%) and the least was observed with chloramphenicol (0%) as show in Table 3.

Results of Biofilm binding activity of S. aureus by tissue culture plate assay

Results of biofilm binding activity of *S. aureus* indicated that: 2 isolate showed strong biofilm activity, 3 isolates showed moderate binding activity, and 2 isolate showed weak/non biofilm producer as shown in Table 4.

Genotypic detection of enterotoxins and integron by PCR

Enterotoxins were detected in the 7 strains (100%) as follow; *SEA* and *SEC* and *SEE* were not detected in any isolate 0%, *SEB* and *SED* were detected in 4 isolates (9.1%) as shown in Figs. 1, 2 and 3.

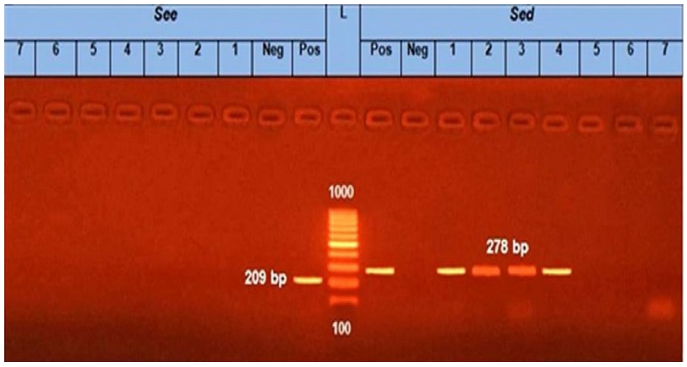

Fig. 1. Ethidium bromide-stained Agarose gel electrophoresis (1.5%) of the amplified *see* (left) and *sed* (right) gene of the isolated *S. aureus*: Lane 5: DNA molecular weight ladder (100bp ladder). P: positive control; N: negative control which is (Nuclease free water). Lanes 1, 2, 3, 4, indicate positive results for *sed* gene (specific band of 278 bp). Lanes 1,2,3,4,5,6,7: negative for *see* gene.

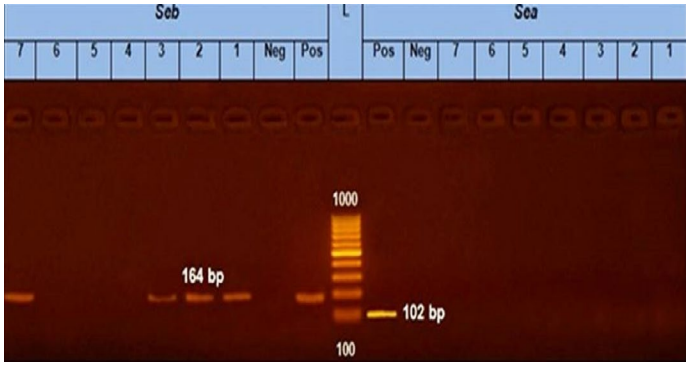

Fig. 2. Ethidium bromide stained Agarose gel electrophoresis (1.5%) of the amplified *seb* (left) and sea (right) gene of the isolated *S. aureus*: Lane 5: DNA molecular weight ladder (100bp ladder). P: positive control; N: negative control which is (Nuclease free water). Lanes 1, 2, 3, 7, indicate positive results for *seb* gene (specific band of 164 bp). Lanes 1,2,3,4,5,6,7: negative for sea gene.

Table 3. The antimicrobial sensitivity patterns of 7 *S. aureus* isolates was determined against 12 antimicrobial agents.

Antimicrobial agent	S		I		R	
	NO	%	NO	%	NO	100
Oxytetracycline (OT)	-	-	-	-	7	100
Amoxicillin/Clavulinic acid (A/C)	-	-	2	28.57	5	71.43
Erythromycin (E)	1	14.29	2	28.57	4	57.14
Ampicillin (AM)	2	28.57	2	28.57	3	42.86
Sulphamethoxazol/Trimethprim (SXT)	3	42.86	1	14.29	3	42.86
Gentamicin (G)	3	42.86	1	14.29	3	42.86
Tetracycline (TE)	4	57.14	-	-	3	42.86
Kanamycin (K)	5	71.43	-	2	3	28.57
Cephalotin (CN)	1	14.29	4	57.14	2	28.57
Norfloxacin (NOR)	4	57.14	2	28.57	1	14.29
Chloramphenicol (C)	6	85.7	1	14.29	-	0
Doxycycline (DO)	7	100	-	-	-	0

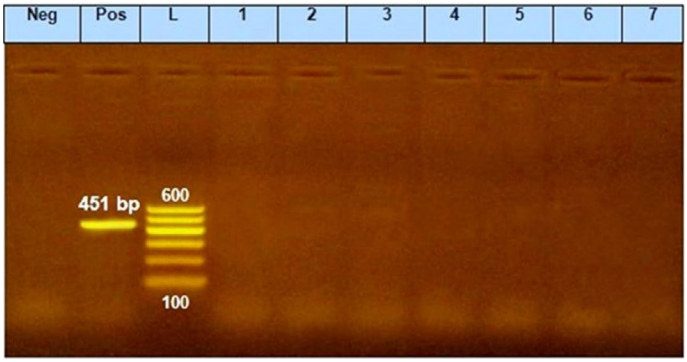

Fig. 3. Ethidium bromide stained Agarose gel electrophoresis (1.5%) of the amplified *sec* gene of the isolated *S. aureus*: lane 5: DNA molecular weight ladder (100bp ladder). P: positive control; N: negative control which is (Nuclease free water). Lanes 1,2,3,4,5,6,7: negative for *sec* gene.

Seven isolates were selected for detection of integron

All the selected isolates were MDR isolates, 6 (10%) showed the presence of integron molecularly with degenerate primers designed to hybridize to conserved regions of integron encoded integrase genes *intI1*, *intI2* and *intI3* using *hep35* and *hep36* genes, the isolate number (1) which was negative as in Fig. 4. Finally, the occurrence of enterotoxin genes and integron and biofilm formation in 7 strains are illustrated in Table 5.

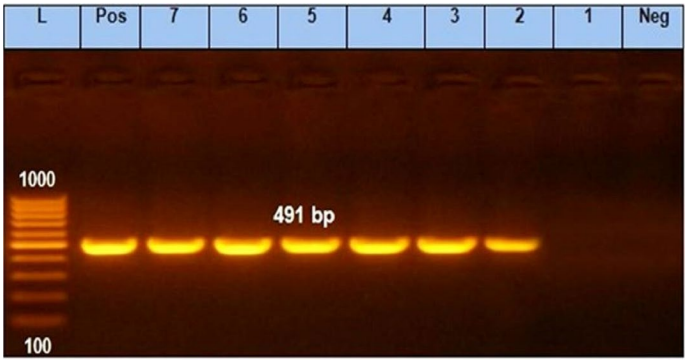

Fig. 4. Ethidium bromide stained agarose gel electrophoresis containing PCR products along with 100bp DNA ladder (lane M). P: positive control; N: negative control which is (Nuclease free water). *S. aureus* isolates (lanes 2,3,4,5,6,7) show positive amplicon at size of 491 bp. Only isolate 1 is negative.

DISCUSSION

Arthritis in chickens is associated with great losses in broiler due to decreased feed intake as the animal becomes unable to walk to obtain food and water which leads to severe losses of weight and sometimes mortalities (Sullivan, 1994), This makes it a need to understand the disease, It´s causes and pathogenicity. Bacterial arthritis in poultry commonly occurred after septicemia or localized infection to the joints is reported to be associated with many bacterial agents including *Staphylococcus* and *Escherichia* (Mohan *et al*., 2001).

Table 4. Results of pathogenic *Staphylococcus aureus* ability to form biofilm.

Sample number	Degree of biofilm formation
Sample (1)	Weak
Sample (2)	Moderate
Sample (3)	Strong
Sample (4)	Weak
Sample (5)	Moderate
Sample (6)	Strong
Sample (7)	Moderate

In this study out of the 70 samples of chickens from different farms, 37 samples were positive for pathogenic *Staphylococcus aureus* (confirmed by positive coagulase test) at a percentage of 52.8%. Rasheed (2011) isolated *Staphylococcus aureus* from poultry agreed this results in a percentage of 50.98%, and El-Masry *et al*. (2015) also reported *S. aureus* at 15.2% 14/92. Same results were recorded by Adayel (20050 and Mutalib *et al*. (1983). The obtained results were higher than that described by Tawfik *et al*. (2015) who reported that out of 40 samples from chickens 10 (25%) were positive for *Staphylococcus aureus* and that of Mamza *et al*. (2010), who isolated *Staphylococcus aureus* from chickens at a percentage of 18.8%. Nazia *et al*. (2015) concluded that *S. aureus* is responsible for septic arthritis in the majority of both commercial broilers and layers, with higher prevalence in hock joints. Furthermore, layers (64.00%) showed a slightly reduced prevalence of *S. aureus* as compared to broilers (68.00%), with no incidence of the causative organism in footpad swellings/injuries.

Additional insertional DNA sequences on the staphylococcal cassette chromosome allow for the inclusion of additional antimicrobial resistance indicators (Liu, 2009). These insertional sequences explain why many methicillin resistant. Staphylococcal organisms have a remarkable ability to become resistant to antimicrobials, as evidenced by the acquisition of drug resistance genes soon after the organisms were exposed to new antimicrobials (Enright *et al*., 2002). The evolution of multidrug is influenced by a wide range of mobile genetic elements. In particular, integrons comprise a substantial proportion of these elements and are often found in plasmids and/or transposons that enhance spread of resistance genes. Six *S. aureus* isolate from 7 examined isolates proved to be positive for integron. The obtained result was higher than that reported by Hosseini *et al*. (2020) and Goudarzi *et al*. (2019), who revealed the presence of integron 1, integron 2 in *Staphylococcus* isolates with percentages of (39.6%,3.7%; 34.1%,14.3%) respectively. This result agrees with Li and Zhao (2018) who revealed the presence of integron 1, integron 2 in *Staphylococcus* isolates with percentages of 85.1%, and 0% respectively. On the other hand, Ammar *et al*. (2016) and Al-Ashmony *et al*. (2016) revealed that none of *S. aureus* investigated in their studies harbored class 1 integrons.

Table 5. The occurrence of enterotoxin genes and integron and biofilm formation in 7 strains are illustrated.

Sample	Enterotoxin producing genes					*Integrase*	*Biofilm formation*	Antimicrobial resistance profile	MAR index
	Sea	*Seb*	*Sec*	*Sed*	*See*				
1	-	+	-	+	-	-	Weak	(OT), (A/C), (E), (AM), (SXT), (G), (TE), (CN).	0.67
2	-	+	-	+	-	+	Moderate	(OT), (A/C), (E), (AM), (SXT), (G), (K).	0.58
3	-	+	-	+	-	+	Strong	(OT), (A/C), (AM), (TE), (K).	0.42
4	-	-	-	+	-	+	Weak	(OT), (A/C), (E), (G), (NOR).	0.42
5	-	-	-	-	-	+	Moderate	(OT), (SXT), (TE), (CN).	0.33
6	-	-	-	-	-	+	Strong	(OT), (E), (TE).	0.25
7	-	+	-	-	-	+	Moderate	(OT), (A/C).	0.17

The differences in the prevalence of integron genes can be due to the differing geographic regions, the bacteria strains, or the indiscriminate use of antibiotics.

A total of 7 samples of positive *S. aureus* were introduced to antibiotic susceptibility test, biofilm and enterotoxin detection. The antibacterial sensitivity pattern of *Staphylococcus aureus* against 12 commercially available antibiotic discs showed 100 % resistance to oxytetracycline followed by Amoxicillin (71.43%), Erythromycin (57.14%), Norfloxacin (14.29%), Tetracycline (42.86), Sulphamethoxazole (42.86%), Gentamicin (42.86%), Ampicillin (42.86%), kanamycin (28.57), cephatotin (28.57), doxycycline (0%) and the least was observed with chloramphenicol (0%) But this result disagreed with the results of Onwubiko and Sadiq (2011) whose isolates showed (7.6%) resistance to Gentamycin, Chloramphenicol (38.1%) but Amoxicillin (69.3%), Erythromycin (47.6%) are near the result. Also, it was differed from result of Magnet *et al*. (2013) who recorded resistant 100% to Gentamicin, but Chloramphenicol (23.57%) near the result. The result agreed with Islam *et al*. (2016) whose result showed the highest resistance to Ampicillin (100%), oxytetracycline (99.17%), Amoxicillin (86.66%), but Gentamycin (13.33%) showed the least resistance. El-Masry *et al*. (2016) showed highest resistance to kanamycin (100%) but agreed with my result in erythromycin (86%), chloramphenicol (73%), gentamycin (40%), and Sulfamethoxazole (46%). Biofilms are organized colonies of bacterial cells that present in a self-produced matrix of extracellular polymeric substances (EPSs), distinguished by a change in gene expression and phenotype Donlan, (2000). Staphylococci are recognized as the most frequent causes of biofilm-associated infections Vuong *et al*., (2004). In this study, two isolates showed strong biofilm activity, three isolates showed moderate binding activity and two isolates showed weak/non biofilm producer. Hazariwala *et al*., (2002) stated that the *S. aureus* was isolated from 11 out of 23 examined samples of minced turkey meat (48%). Using the primers for enterotoxin genes A to C, 4 of the 11 isolated *S. aureus* strains showed a positive result in the PCR. Three of the isolates represented the *SEB* gene and remaining one the *SEC* gene. Hafez *et al*., (2018) found that *S. aureus* could be detected in (11) samples with a percentage of 18.3%. Multiplex PCR was used to investigate the presence of enterotoxins genes (*sea*, *seb*, *sec*, sed, and *see*) in isolated strains. Out of 11 isolates of *S. aureus*, 6 (54.5%) enterotoxins genes detected and represented as one isolate (9.0%) was positive for *Sea*, 2 (18.1%) were positive for *Sec* and 3 (27.2%) were positive for *See* gene.

CONCLUSION

The present study highlights the prevalence of MDR *Staphylococcus aureus* strains in poultry meat. Poultry resistant *S. aureus* isolates are likely to be both enterotoxigenic and biofilm producers. To the best of the author' knowledge, this is the first study in Egypt to report the occurrence of *S. aureus* isolate that carry a class 1 integron gene cassette which confers antibiotic resistance *S. aureus*.

CONFLICT OF INTEREST

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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