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Research Note

Molecular Characterization and Antimicrobial Resistance Profile of Methicillin-Resistant *Staphylococcus aureus* in Retail Chicken

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ABSTRACT

The emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) in food-producing animals is of increasing interest, raising questions about the presence of MRSA in food of animal origin and potential sources of transmission to humans via the food chain. In this study, the prevalence, molecular characterization, virulence factors, and antimicrobial susceptibility patterns of MRSA isolates from 200 retail raw chicken samples in Egypt were determined. MRSA was detected by positive amplification of the *mecA* gene in 38% (76 of 200) of chicken samples analyzed. This represents a potential public health threat in Egypt, as this contamination rate seems to be the highest among other studies reported worldwide. Furthermore, genes encoding α-hemolysin (*hla*) and staphylococcal enterotoxins (*sea, seb,* and *sec*) were detected in all of the 288 MRSA isolates. Nonetheless, none of the strains tested carried *tst,* the gene encoding toxic shock syndrome toxin 1. Antimicrobial resistance of MRSA isolates was most frequently detected against penicillin (93.4%), ampicillin (88.9%), and cloxacillin (83.3%). These results suggest that retail chicken might be a significant potential source for transmission of multidrug-resistant and toxigenic *S. aureus* in Egypt. This underlines the need for stricter hygienic measures in chicken production in Egypt to minimize the risk of transmission of these strains to consumers. To the best of our knowledge, this is the first study that reports the isolation and molecular characterization of MRSA in retail chicken samples in Egypt.

Staphylococcus aureus is a major foodborne pathogen causing outbreaks of food poisoning worldwide *(3, 5)*. The symptoms of staphylococcal food poisoning are nausea, violent vomiting, and abdominal cramping, with or without diarrhea. The onset of symptoms is fast (30 min to 8 h), and spontaneous remission is frequently observed after 24 h *(23)*. *S. aureus* is a part of the normal flora of the skin and mucous membranes of humans and warm-blooded animals and has frequently been isolated from various food products *(20)*. The presence of methicillin-resistant *S. aureus* (MRSA) or its enterotoxins in food is usually indicative of poor hygiene during food production *(2)*. Foodborne contamination of retail chicken likely occurs via food handlers and asymptomatic carriers during the slaughtering and processing of poultry *(13)*.

Treatment of infections caused by *S. aureus* has been further complicated by antimicrobial resistance in bacteria, particularly MRSA *(28)*. MRSA represents those *S. aureus* strains that have acquired the *mecA* gene, encoding penicillin-binding protein 2a (PBP2a), which mediates resistance to methicillin and all other β-lactam antibiotics and therefore represents a public health threat *(14)*. These bacteria are also frequently resistant to most of the commonly used antimicrobial agents *(26)*. The extensive use of antibiotics in humans and animals has increased the emergence of MRSA *(8)*. In Egypt, where antibiotic misuse in animals and poultry production is very common, there are no data available about MRSA in food of animal origin.

Several virulence factors are responsible for the symptoms and severity of infections caused by *S. aureus*. These factors include hemolysins (alpha, beta, gamma, and delta), leukocidin, toxic shock syndrome toxin 1 (TSST-1), and staphylococcal enterotoxins (SEs) *(12)*. SEs are emetic toxins that are recognized agents of staphylococcal food poisoning worldwide *(12)* and are also responsible for other types of infections with shock as a sequela in humans and animals *(27)*. SEs are highly resistant to heat and are considered to be more heat resistant in food products than in a laboratory culture medium *(6)*. Moreover, the proteolytic enzymes trypsin, pepsin, and renin have no effect on SEs, enabling their passage through the gastrointestinal tract without loss of biological activity *(18)*.

The aim of this study was to gain insight into the prevalence of MRSA in retail chicken in Egypt and to characterize the strains isolated during this study using molecular analysis of marker genes (*nuc, coa,* and *mecA*) and virulence genes (*hla, sea, seb, sec,* and *tst*), as well as antimicrobial resistance patterns.

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TABLE 1. *Primer sets for PCR amplification of various genes specific for molecular identification of* Staphylococcus aureus

Target gene	Primer direction and sequence[a]	Amplicon size (bp)	Source
nuc	F: 5′-GTGCTGGCATATGTATGGCAATTG-3′ R: 5′-CTGAATCAGCGTTGTCTTCGCTCCAA-3′	660	This study
coa	F: 5′-TAGGCGCATTAGCAGTTGCATC-3′ R: 5′-CCAGCCGTAGTTTTAACCTCTTG-3′	1,000	This study
mecA	F: 5′-GATTGGGATCATAGCGTCA-3′ R: 5′-CAGTATTTCACCTTGTCCG-3′	1,200	This study
hla	F: 5′-CCGGTACTACAGATATTGGAAGC-3′ R: 5′-GGTAATCATCACGAACTCGTTCG-3′	744	This study
sea	F: 5′-TGCAGGGAACAGCTTTAGGCAA-3′ R: 5′-GATTAATCCCCTCTGAACCTTCC-3′	500	This study
seb	F: 5′-CCTAAACCAGATGAGTTGCACAAAGCG-3′ R: 5′-TCCTGGTGCAGGCATCATGTCATA-3′	600	This study
sec	F: 5′-GCCAGATGAGTTGCACAAATC-3′ R: 5′-CCACCTGTAACTTTACCTAC-3′	300	This study
tst	F: 5′-CGTAAGCCCTTTGTTGCTTG-3′ R: 5′-CCACCCGTTTTATCGCTTGAAC-3′	543	This study

[a] F, forward; R, reverse.

MATERIALS AND METHODS

Collection of samples. A total of 200 retail raw chicken samples (50 each of whole chicken carcasses, drumsticks, chicken gizzards, and chicken livers) were purchased on 10 occasions during the period of July to November 2012 from different supermarkets and poultry butcher shops distributed in Mansoura city, Egypt. Each sample was packaged individually into a clean polyethylene bag, marked, and transferred immediately in an ice box to the laboratory of Food Hygiene and Control Department, School of Veterinary Medicine, Mansoura University, Egypt, for conventional bacteriological analysis.

Isolation, enumeration, and identification of *S. aureus*. The isolation, enumeration, and identification of *S. aureus* were performed according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual (4)*. Briefly, a 25-g amount was aseptically excised by sterile scalpel from each individual sample collected. Samples were taken either from the outer skin in the case of whole chicken carcasses, skin plus muscle in the case of drumsticks, or giblet tissue in the case of gizzards or livers. Samples were transferred into a sterile homogenizer containing 225 ml of Butterfield's phosphate buffer diluent and homogenized for 2 min to provide a homogenate with an original dilution of 1:10. Serial 10-fold dilutions were prepared in sterile test tubes. From the selected dilutions, 0.2-ml amounts were transferred and evenly spread in duplicate onto duplicate plates of Baird-Parker selective agar (Oxoid CM275) supplemented with egg-yolk tellurite emulsion. Inoculated plates were incubated at 35°C for 48 h. Colonies exhibiting typical morphology (grey black to jet black, circular, smooth, convex, 2 to 3 mm in diameter, with a narrow white entire margin surrounded by a zone of clearing extending 2 to 5 mm in the opaque medium) were presumed to be *S. aureus*. The total *S. aureus* count per gram of the examined sample was then calculated and recorded. On plates containing 20 to 200 colonies, the top part of 5 suspected colonies was picked up and inoculated into test tubes containing 5 ml of sterile brain heart infusion broth (Oxoid CM225). The inoculated tubes were incubated at 37°C for up to 24 h for further confirmation based on Gram staining, coagulase test, catalase test, carbohydrate fermentation, and thermostable nuclease production.

Preparation of genomic DNA. Genomic DNA was extracted from tested strains using the Maxwell 16-cell DNA purification kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Genomic DNA from *E. coli* K-12 DH5α strain was used as a negative control template for PCR analyses. DNA samples were sent to Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Hokkaido, Japan, for molecular characterization and sequencing.

Molecular characterization. All of the 288 *S. aureus* strains isolated from the examined chicken products were screened by PCR for nuclease *(nuc)* and coagulase (*coa*) genes (*S. aureus* species-specific determinants) and *mecA* (methicillin resistance determinant). Simultaneous amplification of the three genes was used for MRSA confirmation. Confirmed samples were used as targets for detection of five selected virulence genes, *hla, sea, seb, sec,* and *tst,* which encode α-hemolysin, SEs A, B, and C, and TSST-1, respectively. The primer sets used for PCR amplification of the target genes are listed in Table 1. The primers were synthesized by Hokkaido System Science Co. Ltd. (Hokkaido, Sapporo, Japan). PCR was performed using a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA) in a 20-μl reaction mixture containing 1.6 μl of *S. aureus* DNA template, 1 μl (6 pmol) each of the forward and reverse primer, 4 μl of deoxynucleoside triphosphates (2 mM), 0.4 μl KOD FX Neo DNA polymerase enzyme (1.0 U/μl), 10 μl of 2× PCR buffer for KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan), and 2 μl of PCR-grade water. After an initial denaturation at 94°C for 2 min, 35 amplification cycles consisting of denaturation at 98°C for 10 s, annealing at 58°C for 30 s, and extension at 68°C for 1 min per kbp were performed, followed by a final extension at 68°C for 7 min. Amplicons were separated by subjecting 3-μl aliquots to agarose (1.2%) gel electrophoresis for 30 min at 100 V followed by a 20-min staining in ethidium bromide solution. The separated PCR products were then visualized under UV light and photographed.

DNA sequencing. DNA sequencing was performed for confirmation of the amplified genes on an ABI Prism 3100 automated sequencer (Applied Biosystems) using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied BioSystems) according to the manufacturer's instructions. Primers used for sequencing were the same as used for DNA amplification.

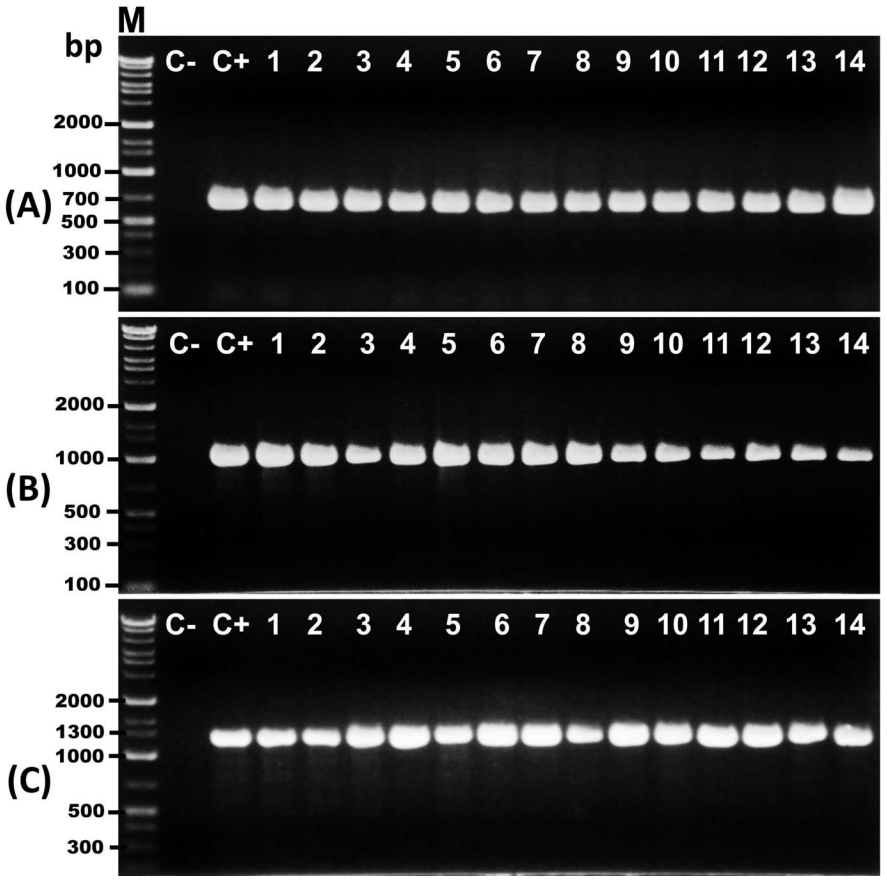

FIGURE 1. *Representative agarose gel electrophoresis of PCR amplicons of the marker genes identified in MRSA isolates from different chicken samples. Amplified bands of the expected sizes of 660 bp for* nuc *(A), 1,000 bp for* coa *(B), and 1,200 bp for* mecA *(C) were visualized under UV light. Lanes M, DNA marker (Gene Ladder Wide 1) used as the reference for fragment size; lanes C-,* E. coli *K-12 DH5α as a negative control strain; lanes C+,* S. aureus *ATCC 43300; lanes 1 to 14, representative strains positive for the target genes.*

Nucleotide sequence data were then analyzed using GENETYX-MAC software, version 12 (GENETYX Corp., Tokyo, Japan).

Antimicrobial susceptibility. Antimicrobial susceptibility patterns for recovered *S. aureus* isolates were determined by the agar disk diffusion method using Mueller-Hinton agar (Oxoid CM0337) supplemented with 5% defibrinated horse blood in accordance with the Clinical and Laboratory Standards Institute guidelines *(10)*. The antimicrobials tested were penicillin (10 IU), amoxicillin (30 μg), ampicillin (10 μg), tetracycline (30 μg), streptomycin (10 μg), cloxacillin (5 μg), erythromycin (15 μg), rifampin (5 μg), chloramphenicol (30 μg), netilmicin (30 μg), ciprofloxacin (5 μg), amikacin (30 μg), gentamicin (10 μg), vancomycin (30 μg), and sulfamethoxazole-trimethoprim (25 μg). The results were recorded and interpreted after 24 h of incubation at 35°C according to the National Committee for Clinical Laboratory Standards guidelines (http://antimicrobianos.com.ar/ATB/wp-content/uploads/2012/11/M100S22E.pdf).

RESULTS AND DISCUSSION

In the present study, we isolated 288 MRSA strains from the positive chicken samples. Ninety-two of these isolates were derived from whole chicken carcasses, 60 from drumsticks, 114 from chicken gizzards, and 22 from chicken livers. All of the 288 isolates identified through conventional microbiological screening were confirmed by amplification of the *nuc* and *coa* genes. These genes were used as target markers to identify *S. aureus* at the species level. The *nuc* gene encodes the thermonuclease enzyme and has been used as a potential genetic marker for rapid diagnosis of *S. aureus* infection. The staphylocoagulase gene *coa* is a marker for discriminating coagulase-positive *S. aureus* from other less pathogenic coagulase-negative staphylococci *(33),* and staphylocoagulase has been considered to be one of the extracellular virulence factors mediating immune evasion *(33)*. Expression of the *coa* gene is thought to enhance bacterial growth and promote infection through escape from host defense mechanisms *(1)*. For MRSA confirmation, the *S. aureus* isolates recovered were tested for the presence of the *mecA* gene. This gene is responsible for resistance to methicillin and other β-lactam antibiotics and has been used as the "gold standard" marker to identify MRSA *(31)*. Using this molecular-typing scheme, *S. aureus* was detected in 38% (76 of 200) of the chicken samples analyzed by amplification of *nuc* and *coa* genes (Fig. 1A and 1B). Interestingly, all samples were confirmed to be MRSA by amplification of the *mecA* gene (Fig. 1C).

Sequence analyses of the amplified genes were carried out, and the resultant sequences were submitted to the GenBank database for homology search using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), which showed 100% identity with the corresponding gene sequences of *S. aureus* accessible in GenBank. Therefore, the overall MRSA contamination rate in this study was 38% (76 of 200) of retail chicken samples tested. Chicken gizzards were the most frequently contaminated samples, with a contamination rate of 56% (28 of 50), followed by whole chickens (44%; 22 of 50), while drumsticks and chicken livers showed relatively lower contamination rates of 28% (14 of 50) and 24% (12 of 50), respectively. Several studies have been conducted in

TABLE 2. Staphylococcus aureus *counts in chicken samples and numbers of samples that reached the maximal permissible limit*

Product	No. of samples tested	*S. aureus* count (log CFU/g)			No. (%) of samples that exceeded the maximal limit[a]
		Minimum	Maximum	Mean	
Whole chickens	50	2.9	3.93	3.16	8 (16)
Drumsticks	50	3.11	3.69	2.89	5 (10)
Gizzards	50	3.0	4.6	3.54	10 (20)
Livers	50	2.0	3.26	2.2	0 (0)
All products	200	2.0	4.6	3.17	23 (11.5)

[a] The maximal permissible limit according to the International Commission on Microbiological Specifications for Foods *(19)* is 4 log CFU/g.

different countries to screen retail chicken meat and chicken products for the presence of MRSA. A study in Germany *(13)* identified MRSA in 25% (6 of 24) of fresh chicken meat and 21% (4 of 19) of chicken meat products. Another study in The Netherlands *(11)* revealed that 16% (83 of 520) of chicken samples were positive for MRSA. In Hong Kong, MSRA was recovered from 7.3% of chicken parts examined *(7)*. Very low contamination rates of MRSA in chicken meat samples were recorded in different countries, for instance, 0.3% (3 of 913) in Korea *(24),* 0.45% (2 of 444) in Japan *(22),* 0.68% (1 of 148) in Spain *(25),* 1.2% (3 of 250) in Canada *(34),* and 1.7% (20 of 1,152) in China *(32)*. MRSA was not detected in any of the 460 food samples of animal origin and 100 pooled neck skin swabs from chicken carcasses in Switzerland *(17)*. Likewise, all of the 45 chicken samples tested in Iowa were negative for MRSA *(16)*. Therefore, the contamination rate shown in this study represents the highest rate reported among other studies conducted among retail chicken samples. The difference in prevalence rates among different studies might be attributed to geographical location, sampling strategy (methods of sampling and time of sampling), enrichment techniques used in bacterial isolation, slaughtering methods, different hygienic practices during slaughtering and food handling, and antimicrobial administration and infection prevention practices *(9, 30)*. In Egypt, hygienic standards are often not followed during manipulation of slaughtered and processed chicken, and antimicrobial agents are used irresponsibly for therapy, prophylaxis, and growth promotion in intensively reared chickens, as well as in treating human illness, all of which might have contributed, in part, to the high prevalence of MRSA in chicken samples reported in this study.

Staphylococci can potentially be expected to exist, at least in low numbers, in all food products of animal origin or in those that are handled directly by humans, unless heat-processing steps are applied to effect their destruction *(21)*. In this study, the mean *S. aureus* counts were 3.16, 2.89, 3.54, and 2.2 log CFU/g in tested whole chickens, drumsticks, gizzards, and livers, respectively, with an overall mean count of 3.17 log CFU/g (Table 2). Of the 200 chicken samples tested, 23 (11.5%) exceeded the maximal permissible limit of 4 log CFU/g set for *S. aureus* in chicken *(19)*. These 23 samples comprised 8 (16%), 5 (10%), and 10 (20%) whole chickens, drumsticks, and gizzards, respectively (Table 2). None of the chicken liver samples tested exceeded the limit.

To trace the possible role of MRSA strains in transmission to humans and in causing infection, the isolates recovered were further tested for five virulence genes, including *hla*, *sea*, *seb*, *sec,* and *tst,* encoding α-hemolysin, *S. aureus* enterotoxins *sea, seb,* and *sec,* and TSST, respectively. Interestingly, all of the 288 MRSA isolates were positive for four virulence genes, *hla, sea, seb,* and *sec* (100%) (Fig. 2A through 2D). These four genes were verified by sequence analyses that showed 100% identity with the corresponding gene sequences of *S. aureus* available in GenBank. α-Hemolysin is the most examined toxin of the *S. aureus* cytotoxins. Most *S. aureus* strains produce this toxin, which is toxic to a wide range of mammalian cells. It is particularly active against rabbit erythrocytes and is also dermonecrotic and neurotoxic *(12)*. Many strains of *S. aureus* can produce a variety of SEs, which are the causative agents of staphylococcal food poisoning through consumption of food containing sufficient amounts of one (or more) of these enterotoxin. SEs are heat resistant and may be related to the clinical manifestations of the disease *(6)*. Enterotoxin A is the most common toxin implicated in staphylococcal food poisoning *(15)*. Studies have shown that enterotoxin C is the most thermostable enterotoxin, followed by enterotoxin B and enterotoxin A *(29)*. To the best of our knowledge, this is the first study that reported the coexistence of three SE genes (*sea, seb,* and *sec*) in all of the *S. aureus* isolates recovered from food products or even from clinical samples. On the other hand, the *tst* gene was not detected in any of the MRSA isolates examined (0%; 0 of 76). TSST is responsible for toxic shock syndrome (TSS). TSS is an acute systemic infection with symptoms characterized by fever, arterial hypotension, cutaneous rash, and skin scaling. In spite of its oral toxicity, TSST has not been documented as medically important in food poisoning *(12)*.

All isolates recovered from chicken meat samples were tested for antimicrobial resistance against 15 antimicrobial agents (Table 3). Resistance was most frequently detected against penicillin (93.4%), ampicillin (88.9%), cloxacillin (83.3%), amoxicillin (77.8%), and erythromycin (73.6%), followed by tetracycline (68.4%), streptomycin (52.1%), and to lesser extents, rifampin (39.2%), amikacin (34.4%), and chloramphenicol (31.6%). On the other hand, 252 (87.5%), 222 (77.1%), 205 (71.2%), 203 (70.5%), and 185 (64.2%) isolates were susceptible to vancomycin, sulfamethoxazole-trimethoprim, ciprofloxacin, netilmicin, and gentamicin, respectively. The differences in the resistance patterns found

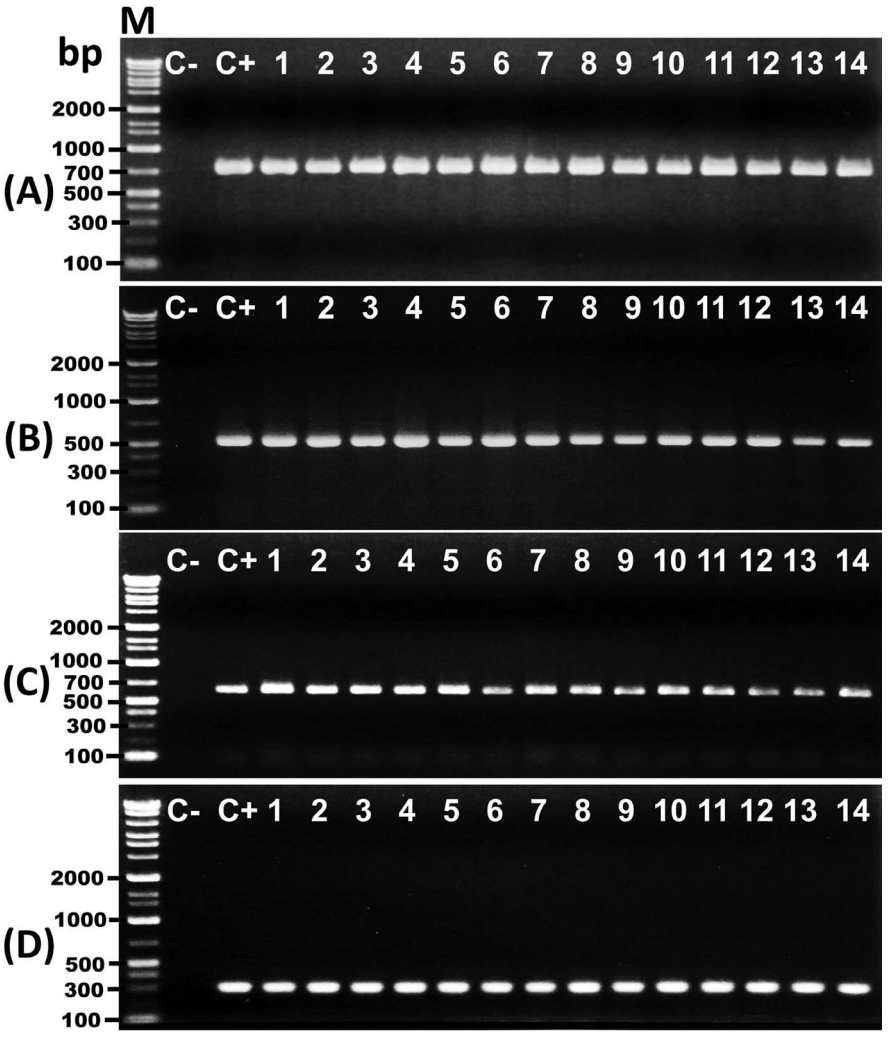

FIGURE 2. *Representative agarose gel electrophoresis of PCR amplicons of the virulence genes identified in MRSA isolates from different chicken samples. Amplified bands of the expected molecular sizes of 744 bp for* hla *(A), 500 bp for* sea *(B), 600 bp for* seb *(C), and 300 bp for* sec *(D) were visualized under UV light. Lanes M, DNA marker (Gene Ladder Wide 1) used as a reference for fragment size; lanes C-,* E. coli *K-12 DH5α as a negative control strain; lanes C+,* S. aureus *ATCC 43300 as a positive control; lanes 1 to 14, representative strains positive for the target genes.*

TABLE 3. *Antimicrobial sensitivity patterns of MRSA isolates from chicken samples*

Antimicrobial (amt used)	No. (%) of MRSA isolates (n = 288) that were:		
	Sensitive	Intermediate	Resistant
Penicillin G (10 IU)	6 (2.1)	13 (4.5)	269 (93.4)
Ampicillin (10 μg)	17 (5.9)	15 (5.2)	256 (88.9)
Cloxacillin (5 μg)	3 (1.04)	45 (15.6)	240 (83.3)
Amoxicillin (30 μg)	25 (8.7)	39 (13.5)	224 (77.8)
Erythromycin (15 μg)	32 (11.1)	44 (15.3)	212 (73.6)
Tetracycline (30 μg)	54 (18.8)	37 (12.8)	197 (68.4)
Streptomycin (10 μg)	83 (28.8)	55 (19.1)	150 (52.1)
Rifampin (5 μg)	144 (50%)	31 (10.8)	113 (39.2)
Amikacin (30 μg)	153 (53.1)	36 (12.5)	99 (34.4)
Chloramphenicol (30 μg)	150 (52.1)	47 (16.3)	91 (31.6)
Gentamicin (10 μg)	185 (64.2)	33 (11.5)	70 (24.3)
Netilmicin (30 μg)	203 (70.5)	37 (12.8)	48 (16.7)
Ciprofloxacin (5 μg)	205 (71.2)	44 (15.3)	39 (13.5)
Sulfamethoxazole-trimethoprim (25 μg)	222 (77.1)	34 (11.8)	32 (11.1)
Vancomycin (30 μg)	252 (87.5)	19 (6.6)	17 (5.9)

in different studies might be attributed to geographical location, locally approved drugs, farm-level management, and misuse or overuse of antibiotics.

In conclusion, this is the first study to report MRSA isolation from retail chicken meat in Egypt. While this study was limited to a relatively small number of samples analyzed, these findings may be crucial in international monitoring of MRSA strains with high virulence potential that are transmitted through retail chicken in Egypt. As a consequence of the high prevalence rate revealed in this study, future studies should examine larger numbers of strains and samples from different retail chicken and human samples in Egypt to delineate their genetic relatedness and to verify that retail chicken meat is a potential vehicle for transmission of MRSA to humans through foodborne transmission, which may help in devising better contamination prevention guidelines for consumer protection.

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