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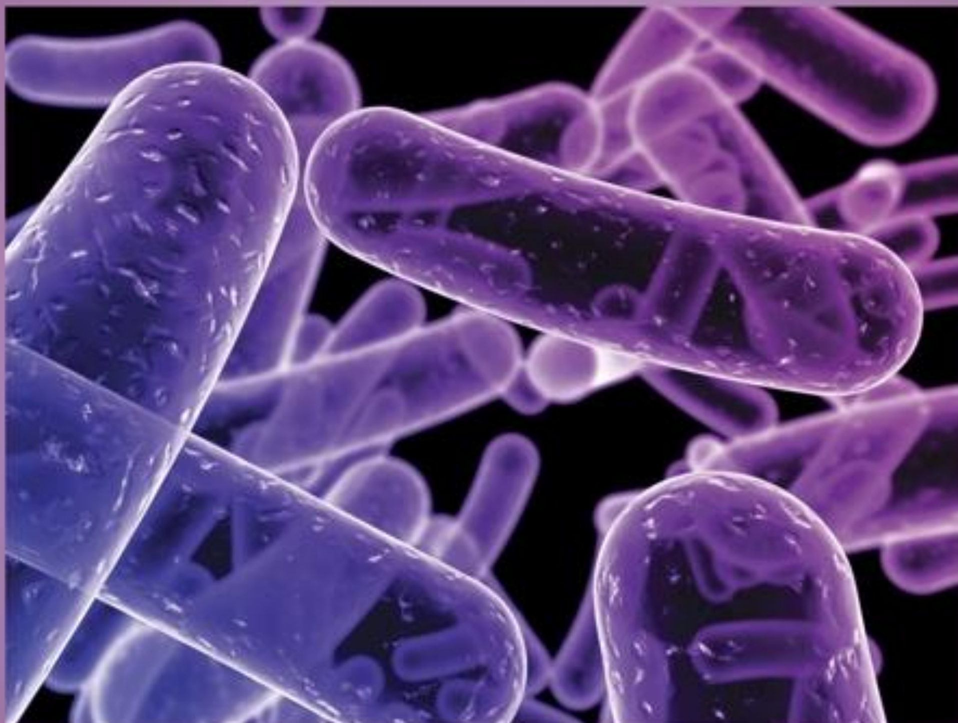

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Prevalence of Multidrug-Resistant *Staphylococcus aureus* in Some Processed Chicken Meat Products

Amer M. M.[1], Islam M. Zakaria[2], Sarah N. Elsayed[1]*, Hazaa M. M.[1] and Amira E. Sehim[1]

1- Department of Botany and Microbiology, Faculty of Science, Benha University
2- Department of Bacteriology Animal Health Research Institute, Gizza

*E.Mail: s.nabil8@yahoo.com - mahmoud.amer@fsc.bu.edu.eg - amira.alsayed@fsc.bu.edu.eg - islamelshereef@yahoo.com

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is one of the most continuously studied foodborne bacteria due to its high pathogenicity, heat-stable enterotoxins production, and its continuous development of multiple antibiotic resistance. Therefore, this study was conducted to investigate prevalence, antibiogram, *mec*A and enterotoxins (SEs) genes of the multidrug-resistant *Staphylococcus aureus* (MRSA) in some processed chicken meat products. A total of 150 random samples, fifty samples each from chicken luncheon, burger, and sausage were collected from some supermarkets in Benha city, Qalubiya governorate, Egypt. The obtained results revealed that among all the examined samples, sausage recorded the highest contamination level with Staphylococcus species (36%) and *S. aureus* (16.0%) with mean counts of 2.1×10^3 and 6.7×10^2 CFU/g respectively followed by luncheon and burger samples. The antimicrobial susceptibility test of 17 *S. aureus* isolates against different antibiotics clearly indicated high susceptibility to Levofloxacin (88.2 %), Ciprofloxacin (82.4%), Gentamicin (70.6%) and Norfloxacin (52.9 %). While, high resistance was observed against Cefotaxime (41.2%) followed by Ampicillin (29.4%), Amoxicillin (29.4%) and Oxacillin (23.5%). Moreover, the staphylococcal enterotoxin-A and *mec*A genes in the examined *S. aureus* isolates were detected by molecular investigation and appeared as positive bands on 102bp and 310bp, respectively. Conclusively, the presence of enterotoxigenic MRSA strains, especially among processed meat products alarms the necessity of strict application of proper hygienic measurements and good manufacturing practices (GMPs) during preparation, handling, and storage of foods.

INTRODUCTION

Staphylococcus aureus is a bacterium characterized by its cluster-shaped arrangement, Gram-positive cocci that have been reported as the main causative agent of various clinical diseases globally.

Infections caused by this bacterium are common in the community and hospital-acquired conditions. Unfortunately, its treatment remains challenging due to the development of multi-drug resistant (MDR) strains (Taylor and Unakal, 2020). *S. aureus* has virulent aggravating characteristics, entero-toxin production and antimicrobial resistance, besides its proteolytic and lipolytic activity in different temperature conditions, causing food spoilage (Puah *et al.*, 2016).

Staphylococcal β-lactams resistance, including penicillins and cephalosporins, is mainly attributed to the presence of *mecA* gene on one of Staphylococcal cassette chromosomes mec (SCCmec), that encodes penicillin-binding protein 2a (PBP2a) with a low affinity for essentially all beta-lactam antimicrobials resulting in difficult treatment of staphylococcal infections (Thaker *et al.*, 2013). The methicillin-resistant *S. aureus* was reported to be one of the most prevalent nosocomial pathogens globally causing a wide range of food poisoning, pneumonia, post-operative wound infections and nosocomial infections (Turner *et al.*, 2010).

The pathogenicity of *S. aureus* may be referred to as the virulence factors associated with drug resistance and affinity to staphylococcal enterotoxin production (Cheung *et al.*, 2021). Staphylococcal food poisoning (SFP) usually occurred after *S. aureus* contaminated food, replicated and produced extracellular heat-stable enterotoxins that render the food dangerous even though it appears normal (Zeaki *et al.*, 2019). Staphylococcal enterotoxins (SEs) mainly of type-A are responsible for SFP symptoms that appeared as rapid onset of gastro-intestinal troubles especially nausea, vomiting, and diarrhea (CDC, 2018).

In Egypt, among the different food products surveyed, chicken meat products had been recorded to be a significant source of enterotoxigenic and/or MRSA representing a harmful public health hazard potential to the consumers (Abdalrahman *et al.*, 2015).

PCR is a specific and effective method for classifying and identifying isolates of *S. aureus*, which demonstrated increasing resistance against many antibiotics, which has been used as a rapid detection technique of Methicillin-Resistant *Staphylococcus aureus* (MRSA) to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence, that considered as an important concern for both treatment and implementation of infection control policies (Ali *et al.*, 2014).

Therefore, the present study was conducted to investigate the incidence of the pathogenic *S. aureus*, especially multidrug-resistant (MDR) strains in different popular processed chicken meat products (luncheon, burger and sausage) sold in Benha city, Qalubiya governorate, Egypt.

MATERIALS AND METHODS

Sample Collection and Processing:

A total of 150 random chicken meat products (50 samples each from luncheon, burger, and sausage) were collected from different supermarkets and groceries in Benha city, Qalubiya governorate, Egypt. Samples were transferred to the laboratory under complete aseptic conditions in an icebox with undue delay and examined for bacteriological and molecular detection of the incidence of *S. aureus* and MRSA strains contamination.

Isolation and Identification of *Staphylococcus aureus:*

Twenty-five grams of the examined samples of meat products were aseptically mixed with 225mL of 0.1% sterile peptone water for 1-2 min to give an initial dilution of 10^{-1}, from which decimal serial dilutions were prepared as described by ISO 6887 (2017). Each previously prepared serial dilution (0.1 mL) was spread over a duplicate large Baired Parker agar plate using a sterile glass spreader. The inoculated and control plates were inverted and incubated at 37°C for 48 hours. After which they were examined for colony characters. The developed colonies were enumerated, and the total staphylococcal count/g was calculated**.** The suspected colonies of *S. aureus* (shiny black with clear

zone extending into opaque medium) were enumerated and *S. aureus* count/g was calculated ISO (6888-1:1999, A1:2003). The isolates were Gram stained, subjected to catalase test, slide and tube coagulase tests, *S. aureus* latex agglutination assay (Pastorex Staph-plus, Bio-rad) and haemolysis test (inoculating onto Columbia colistin nalidixic acid agar with 5% sheep blood and incubated at 37˚C for 24 hours in ambient air). Isolates phenotypically identified as *S. aureus* were subjected to further species confirmation and molecular characterization at Animal Health Research Institute (AHRI), Egypt.

Phenotypic Detection of Methicillin-Resistant *S. aureus* (MRSA):

Methicillin-resistant *S. aureus* (MRSA) was detected using Oxacillin Resistance Screening Agar Base following the method described by Becker *et al.* (2002). Typical 4-5 colonies of each isolated *S. aureus* strain were inoculated in Brain Heart Infusion (BHI) broth incubated at 37°C for 24 hours then, a loopful from inoculated (BHI) broth was streaked on the surface of oxacillin resistance screening agar base plates (ORSAB; Oxoid Limited, Basingstoke, England) with ORSAB Selective Supplement (SR 195 E) and incubated at 37°C for 24 hours for detection of methicillin-resistant *S. aureus* (MRSA).

Molecular Detection of Enterotoxigenic and Multi-Drug Resistant *S. aureus* Isolates:

Two isolates of the confirmed coagulase-positive multidrug-resistant *S. aureus* strains were sent to the Regional Laboratory for Quality control of Poultry Production (RLQP), Animal Health Research Institute, Egypt; and molecularly examined for the presence of *S. aureus* carrying enterotoxin's genes (types A, B, C, D and E) and *mec*A gene using multiplex and uniplex conventional PCR techniques, respectively.

1.DNA Extraction: DNA was extracted by the enzymatic lysis method following manufacturer instructions of QIAamp DNA Mini Kit (Catalogue no. 51304), in which purified DNA was obtained from pure fresh subculture *S. aureus* isolates overnight on nutrient agar. A loop full of the bacterial colony was picked from an isolate and suspended in 200 μL of sterile distilled water, followed by vortexing with 20 μL QIAGEN protease and 200 μl buffer (AL), and incubated at 56°C for 10 minutes. 200 μL of ethanol 96% was added to the previous suspension and transferred to QIAamp mini spin column (in a 2mL collecting tube), and centrifugated at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 mL collection tube. 500 μL of buffer (AW1) were added, and centrifugated at 8000 rpm for 1 min. The collected mixture was placed in a clean 2 mL collection tube, to which 500 μL buffer (AW2) was added, and centrifuged at full speed (10000 rpm) for 3 min. The QIAamp mini spin column was placed in a clean 1.5 mL microcentrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 μl buffer (AE) was added. The QIAamp mini spin column was incubated at room temperature (15-25˚C) for 1 min, and then centrifugated at 8000 rpm for 1 min, and the supernatant containing the genomic DNA was transferred into a fresh sterile Eppendorf tube and stored at −20°C until to be used for PCR.

2.PCR Detection of Staphylococcal Enterotoxins (SEs) Genes: Primers were used for the detection of SEs genes, as shown in Table 1. DNA amplification was performed using the Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit and the PCR assay was carried out in a total volume of 42 μL of the mixture containing 25 μL Emerald Amp GT PCR master mix (2x premix), 1 μL of each of the gene-specific primers (20 pmol), 8 μL of template DNA, and 25 μL of PCR grade water. Amplification conditions included three steps: initial denaturation at 94°C for 5 min; 35 successful cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 40 sec, and extension at 72°C for 45

sec; and the final extension at 72°C for 10 min.

3.PCR Detection of *mec*A Gene: Primers were used for the detection of *mec*A gene, as shown in Table 1. DNA amplification was performed using the Emerald Amp GT PCR master mix (Takara) Code No. RR310A kit and the PCR assay was carried out in a total volume of 25µL of the mixture containing 12.5µL Emerald Amp GT PCR master mix (2x premix), 1 µL of each of the gene-specific primers (20 pmol), 5μL of template DNA, and 5.5µL of PCR grade water. Amplification conditions included three steps: initial denaturation at 94°C for 5 min; 35 successful cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec; and the final extension at 72°C for 7 min. Cycling conditions during PCR: reaction mix was inoculated with Gel Pilot 100 bp ladder (cat. no. 239035) supplied from QIAGEN (USA).

4.Gel electrophoresis: Twenty µL of each uniplex PCR product and 40 µL of each multiplex PCR product, negative and positive controls were loaded to the gel (1.5%). The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Table 1. Target genes and primer sequences for PCR.

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp	**McClure *et al.*, 2006**
	CCA ATT CCA CAT TGT TTC GGT CTA A		
Sea	GGTTATCAATGTGCGGGTGG	102 bp	**Mehrotra *et al.*, 2000**
	CGGCACTTTTTTCTCTTCGG		
Seb	GTATGGTGGTGTAACTGAGC	164 bp	
	CCAAATAGTGACGAGTTAGG		
Sec	AGATGAAGTAGTTGATGTGTATGG	451 bp	
	CACACTTTTAGAATCAACCG		
Sed	CCAATAATAGGAGAAAATAAAAG	278 bp	
	ATTGGTATTTTTTTTCGTTC		
See	AGGTTTTTTCACAGGTCATCC	209 bp	
	CTTTTTTTTCTTCGGTCAATC		

Antimicrobial Sensitivity Test (AST):

The selected isolates were tested for antimicrobial susceptibility using the antibiotic disk diffusion technique (OXOID) in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines. The isolates were tested against 10 antibiotics belonging to β-lactams, aminoglycoside, quinolones, and sulfonamides classes represented by Amoxicillin (AMX25), Ampicillin (AM20), Cefotaxime (CTX30), Ciprofloxacin (CIP5), Erythromycin (E15), Gentamicin (CN10), Levofloxacin (LEV5), Norfloxacin (NOR10), Oxacillin (OX1) and Trimethoprim/ Sulfamethoxazole (SXT1.25/23.75). The result was interpreted as resistant, intermediate, or susceptible based on the inhibitory zone. The strains displaying resistance to at least three antibiotic classes were considered multidrug-resistant (MDR) (Magiorakos *et al.,* 2012).

Statistical Analysis:

The bacteriological counts of staphylococcus and *S. aureus* were entered into SPSS software (Version 22) (IBM, USA) and subjected to descriptive and ANOVA statistics to determine their association between categorical variables. Statistical significance was accepted at $p < 0.05$.

RESULTS

prevalence of Staphylococcus Species and *S. aureus* in Different Chicken Meat Products:

Results demonstrated in Table (2) clearly indicated that, out of all tested samples, sausage exhibited the highest total staphylococcal counts ($2.1x10^3$ CFU/g), followed by luncheon ($1.3x10^3$ CFU/g) and burger ($7.3x10^2$ CFU/g) with the incidence of 36, 24 and 20%, respectively.

Table 2. Total staphylococcus species in the examined chicken meat products.

Product	+ve samples		Counts (CFU/g)		
	No.	%*	Min.	Max.	Mean ± SE
Luncheon	12	24	$5.4x10^2$	$1.9x10^3$	$1.3x10^3 \pm 0.12x10^{3b}$
Burger	10	20	$1.9x10^2$	$1.2x10^3$	$7.3x10^2 \pm 0.6x10^{2b}$
Sausage	18	36	$6.3x10^2$	$3.1x10^3$	$2.1x10^3 \pm 0.26x10^{3a}$
Total	40	26.6**			

(a, b) values within a column with different superscript letters were significantly different at ($P \leq 0.05$).
* Percentage in relation to the total number of each sample (50).
** Percentage in relation to the total number of samples (150)
.

Furthermore, data recorded in Table (3) demonstrated that among the examined samples, *S. aureus* was most commonly detected in sausage (16.0 %), followed by luncheon (10.0%) and burger (8.0%), with mean counts of $6.7x10^2$, $4.5x10^2$ and $3.4x10^2$ and $4.5x10^2$ CFU/g respectively; no significant difference was detected between the different examined samples when $P \leq 0.05$.

Table 3. Prevalence of *Staphylococcus aureus* in the examined chicken meat products.

Product	+ve samples		Count (CFU/g)		
	No.	%*	Min.	Max.	Mean ± SE
Luncheon	5	10.0	$1.0x10^2$	$8.6x10^2$	$3.4x10^2 \pm 0.1x10^{2b}$
Burger	4	8.0	$1.2x10^2$	$9.0x10^2$	$4.5x10^2 \pm 0.3x10^{2b}$
Sausage	8	16.0	$3.5x10^2$	$9.9x10^2$	$6.7x10^2 \pm 0.7x10^{2a}$
Total	17	11.3**			

(a, b) values within a column with different superscript letters were significantly different at ($P \leq 0.05$).
* Percentage in relation to the total number of each sample (50).
** Percentage in relation to the total number of samples (150).

Phenotypic Characterization of MRSA on ORSAB Agar:

Data presented in Table (4) showed that of all tested *S. aureus* isolates, only four isolates (2.6%) were MRSA (intense blue in color on a colorless background) indicating its oxacillin resistance.

Table 4. Characterization of MRSA strains on ORSAB agar.

Product	*S. aureus* isolates		MRSA strains on ORSAB agar	
	No.	%*	No.	%*
Luncheon	5	10.0	1	2.0
Burger	4	8.0	1	2.0
Sausage	8	16.0	2	4.0
Total	17	11.3**	4	2.6**

* Percentage in relation to total number of each sample (50).
** Percentage in relation to total number of samples (150).

Antibiogram Assay of *S. aureus* Isolates:

Antimicrobial susceptibility test of 17 *S. aureus* isolates to 10 antimicrobial agents belonging to four classes revealed that out of 17 isolates, only 4 (23.5%) were resistant to Oxacillin, while 5 (29.4%) and 7 (41.2%) were resistant to Amoxicillin, and Cefotaxime respectively. On the other hand, 15 (88.2%) showed high susceptibility to Erythromycin and Levofloxacin and 14 (82.4%) were sensitive to Ciprofloxacin as shown in Table (5).

Table 5. Antimicrobial susceptibility test of isolated *S. aureus* strains.

Antimicrobial agents	Disk concentrations	Antibiotic class	Sensitive		Intermediate		Resistant		Result
			No.	%	No.	%	No.	%	
Amoxicillin	25µg	β-Lactam	10	58.8	2	11.8	5	29.4	S
Ampicillin	20 µg	β-Lactam	11	64.7	1	5.9	5	29.4	S
Cefotaxime	30 µg	β-Lactam	9	52.9	1	5.9	7	41.2	S
Ciprofloxacin	5 µg	Quinolones	14	82.4	0	0	3	17.6	S
Erythromycin	15 µg	Aminoglycoside	15	88.2	1	5.9	1	5.9	S
Gentamicin	10 µg	Aminoglycoside	12	70.6	3	17.6	2	11.8	S
Levofloxacin	10 µg	Quinolones	15	88.2	1	5.9	1	5.9	S
Norfloxacin	10 µg	Quinolones	9	52.9	4	23.5	4	23.5	S
Oxacillin	1 µg	β-Lactam	12	70.6	1	5.9	4	23.5	S
Trimethoprim/ Sulphamethoxazol	SXT/25 (1.25/23.75) mcg	Sulfonamides	13	76.5	1	5.9	3	17.6	S

No.: Number of isolates,
%: Percentage in relation to the total number of isolates (17).
R: Resistant, S: Sensitive, IS: Intermediate

Detection of *mec*A and Enterotoxins Genes among *S. aureus* Isolates:

The most resistant isolates were further examined for the presence of *mec*A and enterotoxin genes using specific primers in a conventional PCR assay. The *mec*A gene was detected only in two *S. aureus* isolates. Moreover, these isolates showed the gene encoding for enterotoxin A (*Sea*) while, they were negative for other enterotoxins genes (Seb, *Sec, Sed, See*) as presented in Figure (1).

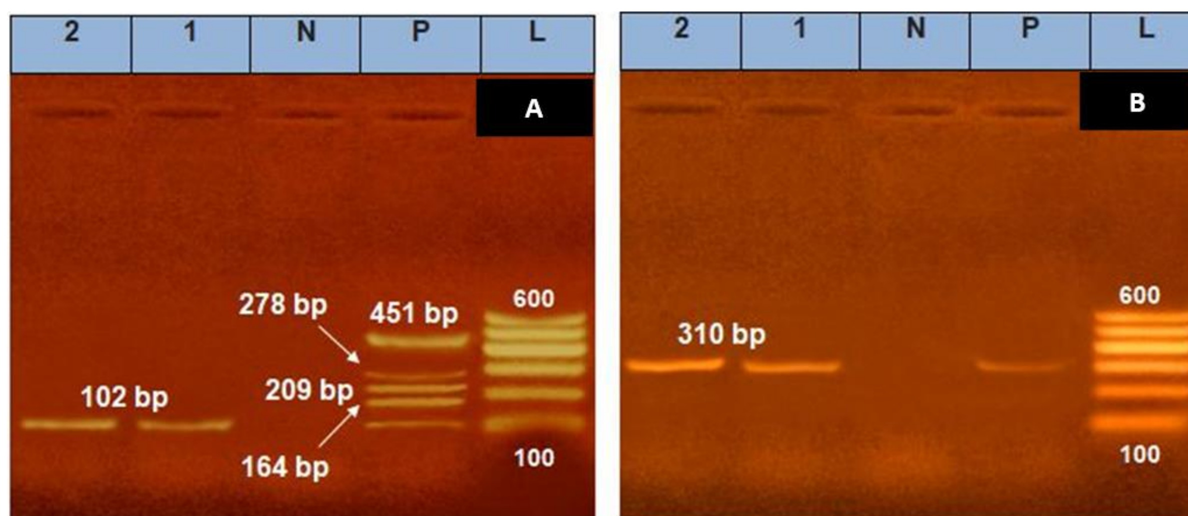

Fig. 1: PCR amplification of SEs (A) and *mec*A genes of *S. aureus* isolates on 1.5% gel electrophoresis**,** Lane L: Ladder (MW 100-600 bp fragments), Lane P: Positive control at 102, 164, 451, 278, 209 and 310 bp of *sea, seb, sec, sed, see*, and *mec*A genes, respectively, Lane N: Negative control (*E. coli* local isolate), Lanes 1 and 2: typical band size of 102 and 310 bp corresponding to *sea* (A) and *mec*A genes(B).

DISCUSSION

For a few decades, *S. aureus* was reported to cause about 25% of all recorded foodborne outbreaks in the USA. Detection of MRSA strains in some food poisoning cases was also attributed to continuous misuse of antibiotics; and however low prevalence of MRSA in food, its hazard comes from difficulties of treating infections due to multi-drug resistance emergence (Bean *et al.*, 1997; Sciezynska *et al.,* 2012; Cha *et al.*, 2014).

In the present study, results revealed that sausage samples recorded the highest incidence and counts of staphylococcus species and *S. aureus*, followed by luncheon and burger, respectively, which may be referred to the improper sanitation of sausage casing, usage of low grades of spices and additives and/or improper storage environment.

Referring to previous records, many local studies were conducted to assess the hygienic quality of chicken meat products. Hosny (2016) demonstrated that 45.7, 48.6 and 54.3% of the examined burger, luncheon and sausage samples were contaminated with staphylococcus sp., from which 14.3, 17.1 and 28.6% were positive for *S. aureus.* Awad (2019) investigated the detection of staphylococcus sp. in luncheon samples with mean counts of 8.9×10^2 CFU/g, where *S. aureus* was detected in 25% of the examined samples with a mean count of 3.6×10^2 CFU/g. Barakat (2020) recorded that mean counts of *S. aureus* in the examined chicken sausage and luncheon samples were 1.6×10^2 and 3×10^2 CFU/g, respectively.

Variations between authors may be attributed to the differences in manufacturing, processing and handling procedures. The presence of *S. aureus* in such food items highlighted preparation, handling, storage, or service faults which may come through cross-contamination, food handlers' faults and the surrounding environment; in addition, spices, equipment, dressings, knives, and other additives are considered as the source of contamination.

Results of the antimicrobial sensitivity test were somewhat agreed with the results recorded by (Bahbah, 2019; Hosny, 2016) they recorded a multidrug resistance of their *S. aureus* isolated from meat and meat products. Most of *S. aureus* isolates were resistant to all β-lactams antibiotics, which is conferred by the *mec*A gene, which codes for an altered penicillin-binding protein (PBP2a or PBP20) that has a lower affinity for binding β-lactams (penicillins, cephalosporins, and carbapenems). This allows resistance to all β-lactam antibiotics and obviates their clinical use during MRSA infections as mentioned by Chambers (2001).

Moreover, results of molecular detection of the presence of MRSA in the examined samples were in good agreement with findings of (Laban, 2018; Morshdy *et al.*, 2018; Seif, 2020 and Gaafar, 2020) they detected staphylococcal enterotoxin-A (*sea*) and *mec*A genes containing *S. aureus* isolate from their examined meat product samples.

Conclusion

The high prevalence of *S. aureus* among the tested samples, mainly in sausage samples, and the presence of the MRSA in processed foods highlighted the necessity of enforcing hygienic practices within meat processing plants. In the future, the molecular and ecological characterization of isolated MRSA strains must be performed to determine the origin of the contamination. Better knowledge of strict hygienic practices during the collection of raw materials, preparation of food, holding, storage and serving must be educated to food handlers.

Conflicts of Interest:

The content of this report solely reflects the opinions of the authors, and we report no conflicts of interest.

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