Bacteriological and Molecular studies on Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolated from chicken meat and its products in Kaliobia Governorate

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A B S T R A C T

The study was performed on 175 random samples of fresh broiler chicken and chicken meat products viz: Chicken burger; chicken luncheon; chicken kofta and chicken sausage (35 for each), collected from different shops at Benha city, kaliobia government. The sample were examined for detection the prevalence of Staphylococci with special reference to *S. aureus* and MRSA, beside the phenotypic characterization of the isolated *S. aureus* strains and detection of their virulence genes in them. The bacteriological examination revealed the isolation of 98 Staphylococcus species including 41 *S.aureus*; 54 *S. epidermidis* and 3*S. chromogenes*. In addition, all 41 isolated *S. aureus* were coagulase positive strains, while, the other isolated Staphylococcal strains (57) were coagulase negative ones. Moreover, the results of SET- RPLA test revealed that 5 *S. aureus* strains out of 10 randomly examined strains were enterotoxigenic and classified according to type of toxin into (3A; 1 B & 1C). The sensitivity tests for the isolated *S. aureus* showed strains indicated high resistance to methicillin followed by oxacillin; Nalidixic acid; Ampicillin; Amoxicillin; Cefotaxime and Tobramycin, respectively. Meanwhile, they were highly sensitive to Gentamycin; Enrofloxacin; Norfloxacin; Lomefloxacin and Ciprofloxacin. PCR results cleared that, *femA; mecA* and *hlb* virulence genes were detected in all 5 *S. aureus* studied strains. Meanwhile, *ica A* was detected in 4studied strains; enterotoxin A (*sea*) was detected in 2 studied strains chicken meat and chicken kofta samples only and leukocidin (*pvl*) virulence gene was detected in 1 studied strain of chicken meat sample.

Keywords: Chicken meat products, bacteriological evaluation, *S. aureus*, MRSA, PCR, virulence factors.

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1. INTRODUCTION

Poultry meat is a common vehicle of *S. aureus* that considered as one of the most important causes of foodborne outbreaks in people (Losito et al., 2005), 2005). Staphylococci are Gram-positive cocci, form grape-like clusters on Gram's stain, non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation. *Thus, S. aureus* is considered the third-most important cause of food-borne disease in the world (Liu et al., 2006; Normanno et al., 2007) and has two aggravating characteristics, toxin production and antimicrobial resistance as well as proteolytic and lipolytic activity at+20 °C, causing meat spoilage (Gundogan and Devren, 2010) . The methicillin-resistant *S. aureus* (MRSA) is mainly attributed to the presence of *mecA* gene, located 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 infections (Pinho et al., 2001; Thaker et al., 2013; Weese et al., 2010). The methicillin-resistant *S. aureus* (MRSA) is known to be one of the most prevalent nosocomial pathogens throughout the world and is capable of causing a wide range of food poisoning, pneumonia, post-operative wound infections and nosocomial infections (Cuny et al., 2010; Hoerlle and Brandelli, 2009).

In recent years, methicillin-resistant *S. aureus* (MRSA) has been identified in domestic animals and animal-derived food products worldwide (Bhargava et al., 2011; Hanson et al., 2011; Saleha and Zunita, 2010). Of the various food products surveyed, chicken and chicken products are widely known to be important reservoir and main source of MRSA in humans (Abdalrahman et al., 2015; Aklilu et al., 2016; Fessler et al., 2011). The pathogenicity of *S. aureus* could be attributed to the virulence factors the bacteria produce. These virulence factors include, intracellular adhesion (*icaA*); toxins (enterotoxins, toxic shock syndrome

toxin-1, Panton-Valentine Leukocidin); hemolysin; coagulase, thus clot blood; protease; hyaluronidase, and staphylokinase (Abdalrahman et al., 2015; Bokarewa et al., 2006; Dinges et al., 2000; Lin and Peterson, 2010). *S. aureus* produce disease when the bacteria contaminate food, produce some enzymes which are implicated with Staphylococcus invasiveness and many extracellular substances some of which are heat stable enterotoxins that renders the food dangerous even though it appears normal and extensive cooking can be killed the bacteria but the toxins may not be destroyed because most of them are gene based i.e. they can be carried on the plasmid (Prescott et al., 2005). The Staphylococcal enterotoxins (SEs) mainly, *sea* is responsible for the symptoms that associated with Staphylococcal food poisoning (Balaban and Rasooly, 2000; Llewelyn and Cohen, 2002). As Egypt has a large chicken production industry, few studies have been applied on *S. aureus*, especially MRSA; in them, that constitutes serious problems for consumers.

Therefore, such study was performed for detection the prevalence of Staphylococci with special reference to *S. aureus* and MRSA, beside the phenotypic characterization of the isolated *S. aureus* strains and detection of some virulence genes.

2. MATERIAL AND METHODS

2.1. Samples collection:

A total of 175 random samples of fresh broiler chicken and chicken meat products viz: Chicken burger; chicken luncheon; chicken kofta and chicken sausage (35 for each), were collected from different shops at Benha city for studying their contamination with Staphylococci.

2.2. Bacteriological examination

Accurately, 25 grams of each sample under examination were prepared for bacteriological examination following (APHA, 2001).

2.2.1. Isolation and identification of Staphylococci strains:

Isolation and identification of Staphylococci strains from different samples according to Quinn et al. (2002) and (Arora, 2003) as follow: One ml of prepared sample was inoculated into nutrient broth and incubated aerobically at 37ºC for 12 hours. A loopful from incubated nutrient broth was streaked on 7% salted nutrient agar and incubated for 24 hours at 37°C. Then the following tests (Oxidase test, Catalase test and Coagulase test) were performed on yellow convex colonies. The colonies that gave (Oxidase –ve, Catalase +ve, KOH 3% -ve and Coagulase +ve & -ve) were taken and cultivated on the following media: Baird Parker agar; Mannitol salt agar; Milk salted agar and Blood agar and incubated for another 48 hours at 37ºC. Suspected colonies (black colonies with yellow halo around them on Baird-Parker agar; yellow colonies surrounded by halo zone on Mannitol salt agar; yellow colonies and turned media to colorless on 7% salted milk agar and white or yellow, smooth round and shiny colonies on blood agar) were picked up and kept in semi-solid agar. The purified isolates of Staphylococci species were morphologically identified by Gram stain and biochemical tests.

2.2.2. In-Vitro anti-microbial sensitivity test:

The isolated *S. aureus* strains were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method (Finegold and Com, 1982).

2.2.3. Detection of enterotoxins producing isolates by SET- RPLA technique (Igarashi et al., 1986).

2.2.4. Virulence genes of S. aureus detection by PCR:

PCR was applied by using 6 sets of primers for detection of 6 virulence genes that may play a role in virulence of *S. aureus*. These genes were factor essential for methicillin- resistance A (*femA*), (*mecA*), leukocidin (*pvl*), β- haemolysin (*hlb*), intra –cellular adhesion (*ica A*) and enterotoxin A (*sea*).It was applied on 5 random isolated *S. aureus* (one isolate from each sample) following QIAamp® DNA Mini Kit instructions (Catalogue no. 51304), Emerald Amp GT PCR mastermix (Takara) with Code No. RR310A and 1.5% agarose gel electrophoreses (Sambrook et al., 1989).

3. RESULTS

3.1. The results of bacteriological examination:

The results of bacteriological examination of examined samples recorded in Table (1) revealed that, a total of 98 (56.0%) isolates of Staphylococcus species were recovered from 175 samples, including 41 *S. aureus* (23.4%); 54 *S. epidermidis*(30.9%) and 3 *S. chromogenes* (1.7%) *S. aureus* were isolated from 41 samples (23.4%); represented as 12 (34.3%) from chicken meat followed by 10 (28.6%) from chicken sausage; 8 (22.9%) from chicken kofta; 6 (17.1%) from chicken luncheon and 5(14.3%) from chicken burger. Meanwhile, *S. epidermidis* were isolated from 54 samples (30.9%); represented as12 (34.3%) from chicken kofta followed by11 from

each samples of chicken meat; chicken burger and chicken luncheon (31.4%) and 9 (25.7%) from chicken sausage. Moreover, 3*S. chromogenes* (1.7%) were isolated from 2 samples of chicken meat (5.7%) and 1 from chicken kofta (2.8%) only. The recovered isolates grow well on different media. *However, S. aureus* showed yellow convex colonies on 7% salted nutrient agar; yellow colonies (with fermentation of mannitol) surrounded by halo zone on mannitol salt agar medium; while on Baird-Parker agar media showed black shiny colonies (due to tellurite reduction) with yellow halos around them on the surface of the medium and some strains also produce a smaller, clear zone around the colonies due to proteolytic activity. At the same time, the same isolates on blood agar showed a white or yellow, smooth round and shiny colonies with beta and alpha hemolysis and on 7% salted milk agar they give yellow colonies and turned media to colorless due to lipase enzyme. Meanwhile, *S. epidermidis* showed white, raised, convex colonies on nutrient agar; black colonies on Baird Parker agar; white colonies (without fermentation of mannitol) on Mannitol salt agar; white colonies on Milk salted agar and smooth round, shiny and non -hemolytic colonies on Blood agar. Moreover, *S. chromogenes* showed black colonies on Baird parker agar; orange colonies (without fermentation of mannitol) on Mannitol salt agar; orange colonies on Milk salted agar and yellow, smooth round, shiny and non-hemolytic colonies on Blood agar. In addition, out of 98 isolated Staphylococcus species strains, 41 strains were coagulase positive, all of them were *S. aureus strains*, and the other isolated Staphylococcal strains (57) were coagulase negative ones.

3.2. The results of SET -RPLA test:

The results of SET -RPLA test revealed that 5 strains out of 10 randomly examined strains (50.0%) were enterotoxigenic and classified according to type of toxin into (3A;1 B & 1C).

3.3. The results of in vitro sensitivity test:

The results of in vitro sensitivity test Table (2) showed that, the isolated *S. aureus* were resistant for Methicillin; Oxacillin (73.2%); Nalidixic acid (65.8%); Ampicillin (63.4%); Amoxicillin (61.0%); Cefotaxime (53.6%) and Tobramycin (43.9%). Meanwhile, they were highly sensitive to Gentamycin (85.3%); Enrofloxacin and Norfloxacin (82.9% for each); Lomefloxacin (73.2%) and Ciprofloxacin (70.7%). Moreover, they were intermediate sensitive to Sulfa-trimethoprim (51.2%); Neomycin (48.8%); Streptomycin (43.9%) and Erythromycin (41.5%).

3.4. PCR results:

PCR results showed that *femA; mecA* and *hlb* virulence genes were detected in all 5 studied strains (100.0%). Meanwhile, *ica A* was detected in 4 (80%) studied strains; enterotoxin A (*sea*) was detected in 2 studied strains chicken meat and chicken kofta samples only (40.0%) and leukocidin (*pvl*) virulence gene was detected in 1 studied strains of chicken meat sample only (20.0%). Moreover, the *femA* gene was amplified in all 5 studied *S. aureus* strains (100.0%)giving product of 132 bp; the *mecA* gene was amplified in all 5 studied *S. aureus* strains (100.0%) giving product of 310 bp; the *hlb* gene was amplified in all 5 studied *S. aureus* strains (100.0%) giving product of 496 bp; the *icaA* gene was amplified in 4(80.0%) *S. aureus* strains giving product of 1315 bp; the *sea* gene was amplified in 2 (40.0%) *S. aureus* strains giving product of 102 bp and the *pvl* gene was amplified in one *S. aureus* strains of chicken meat sample only (20.0%) giving product of 433 bp as shown in Fig. (1-6).

Table (1): Incidence of Staphylococcus species strains isolated from examined samples

Samples	Staphylococcus species							
	S. aureus		*S. epidermidis*		*S. chromogenes*		Total	
	NO.	%	NO.	%	NO.	%	NO.	%
Chicken Meat	12	34.3	11	31.4	2	5.7	25	71.4
Chicken Burger	5	14.3	11	31.4	0	0.0	16	45.7
Chicken Luncheon	6	17.1	11	31.4	0	0.0	17	48.6
Chicken Kofta	8	22.9	12	34.3	1	2.8	21	60.0
Chicken Sausage	10	28.6	9	25.7	0	0.0	19	54.3
Total (175)	41	23.4	54	30.9	3	1.7	98	56.0

Percentage in relation to total number of each sample in each row (35 for each sample &175 for total).

Table (2): In-Vitro anti-microbial Sensitivity test for isolated *S. aureus* strains

Antimicrobial agents	Disk concentrations	Sensitive		Intermediate		Resistant		
		No.	%	No.	%	No.	%	AA
Amoxicillin	25µg	6	14.6	10	24.4	25	61.0	R
Ampicillin	20µg	15	36.6	0	0.0	26	63.4	R
Cefotaxime	30µg	7	17.1	12	29.3	22	53.6	R
Ciprofloxacin	5 µg	29	70.7	4	9.8	8	19.5	S
Enrofloxacin	5 µg	34	82.9	3	7.3	4	9.8	S
Erythromycin	15 µg	14	34.1	17	41.5	10	24.4	IS
Gentamicin	10 µg	35	85.3	4	9.8	2	4.9	S
Lomefloxacin	10 µg	30	73.2	6	14.6	5	12.2	S
Methicillin	5 µg	2	4.9	8	19.5	31	75.6	R
Nalidixic acid	30 µg	2	4.9	12	29.3	27	65.8	R
Neomycin	30 µg	14	34.1	20	48.8	7	17.1	IS
Norfloxacin	10 µg	34	82.9	4	9.8	3	7.3	S
Oxacillin	1µg	3	7.3	8	19.5	30	73.2	R
Streptomycin	S/10	7	17.1	18	43.9	16	39.0	IS
Sulfa-trimethoprim	TMP5	9	22.0	21	51.2	11	26.8	IS
Tobramycin	10 µg	11	26.8	12	29.3	18	43.9	R

No.: Number of isolates. %: Percentage in relation to total number of isolates (41). AA: Antibiogram activity

4. DISCUSSION

So far, comparatively little information is available for Methicillin-resistant Staphylococci, particularly MRSA from poultry in general and in particular for MRSA from poultry in Egypt. Therefore, this study was conducted to throw light over the prevalence of Staphylococci with special reference to *S. aureus* and MRSA in retail raw chicken meat and its common products (burger; luncheon; kofta and sausage) sold in markets in Kaliobia Governorate beside the phenotypic characterization of the isolated *S. aureus* strains and detection of some virulence genes in them.

The results of Staphylococcus species strains isolation from examined samples Table (1) cleared that, a total of 98(56.0%) isolates of Staphylococcus species were recovered from 175 samples, includes 41 *S. aureus* (23.4%); 54 *S. epidermidis* (30.9%) and 3 *S. chromogenes* (1.7%). *S. aureus* were isolated from 41 samples (23.4%); represented as 12 (34.3%) from chicken meat followed by 10 (28.6%) from chicken sausage; 8 (22.9%) from chicken kofta; 6 (17.1%) from chicken luncheon and 5(14.3%) from chicken burger. Meanwhile, S. epidermidis were isolated from 54 samples (30.9%); represented as12 (34.3%) from chicken kofta followed by11 from each samples of chicken meat; chicken burger and chicken luncheon (31.4%) and 9 (25.7%) from chicken sausage. Moreover, 3 *S. chromogenes* (1.7%) were isolated from 2samples of chicken meat (5.7%) and 1 from chicken kofta (2.8%) only. Nearly similar results for *S. aureus* isolation were recorded by Abdalrahman et al. (2015); Ahmed (2015); Akbar and Anal (2013); Aklilu et al. (2016); Citak and Duman (2011); Feßler et al. (2012.); Lim et al. (2010).

The colonial appearance and the biochemical profile of Staphylococci and isolated *S. aureus* was similar to those previously reported such as the fermentation of certain sugars or enzymatic reaction as lipase; extracellular pigmentation production (Staphyloxathine) and Staphylo-coagulase (Ahmed, 2015; Chandrakanth et al., 2010; Quinn et al., 2002). Moreover, out of 98 isolated Staphylococcus species strains, 41 strains were coagulase positive and all of them were *S. aureus* strains and the other isolated Staphylococcal strains (57) were coagulase negative ones (Table, 2). Nearly similar results were obtained by Chandrakanth et al. (2010); Karmi (2013); Momtaz et al. (2013).

The widespread use of antibiotics has undoubtedly accelerated the virulence of *S. aureus*, by acquiring multiple resistance genes, has become able to survive almost all antibiotic families (Stefani and Goglio, 2010). Several workers have reported the occurrence of multidrug resistant *S. aureus* in poultry (Waters et al., 2011). The in-vitro sensitivity tests for the isolated *S. aureus* (Table, 2) showed that, the isolated *S. aureus* were resistant for Methicillin; Oxacillin; Nalidixic acid; Ampicillin; Amoxicillin; Cefotaxime and

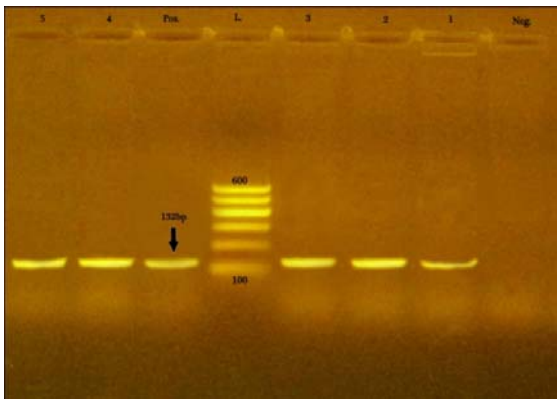

Fig. (1): (*femA*) gene. Lane L: 100-600 bp DNA Ladder. Neg.: Negative control (Listeria reference: NCINB50007) Pos.: Positive control (*S. aureus* reference: ATCC25923 at 132 bp). Lane 1; 2; 3; 4 & 5: *S. aureus* (Positive).

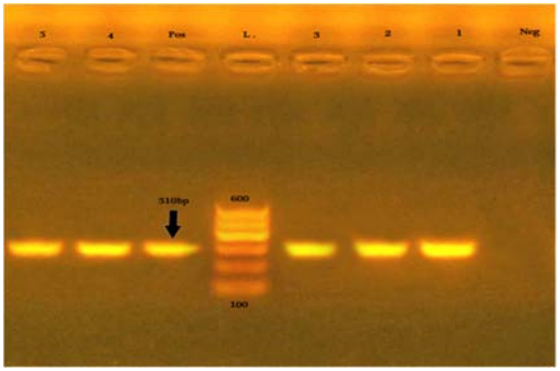

Fig (2): (*mecA*) gene. Lane L: 100-600 bp DNA Ladder. Neg.: Negative control (Listeria reference: NCINB50007). Pos.: Positive control (*S. aureus* reference: ATCC25923 at 310 bp). Lane 1; 2; 3; 4 & 5: *S. aureus* (Positive).

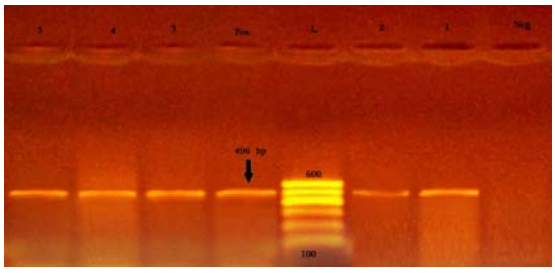

Fig. (3): β- hemolysin (*hlb*) gene. Lane L: 100-600 bp DNA Ladder. Neg.: Negative control (Listeria reference: NCINB50007) Pos.: Positive control (*S. aureus* reference: ATCC25923 at 496 bp). Lane 1; 2; 3; 4 & 5: *S. aureus* (Positive).

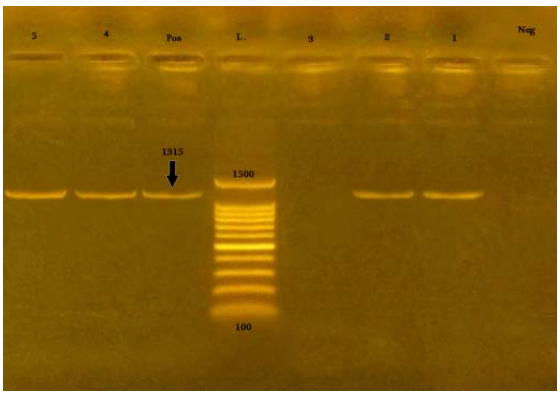

Fig. (4): Intra-cellular adhesion (*icaA*) gene. Lane L: 100-1500 bp DNA Ladder. Neg.: Negative control (Listeria reference: NCINB50007) Pos.: Positive control (*S. aureus* reference: ATCC25923 at 1315 bp). Lane 1; 2; 4 & 5: *S. aureus* (Positive). Lane 3: *S. aureus* (Negative).

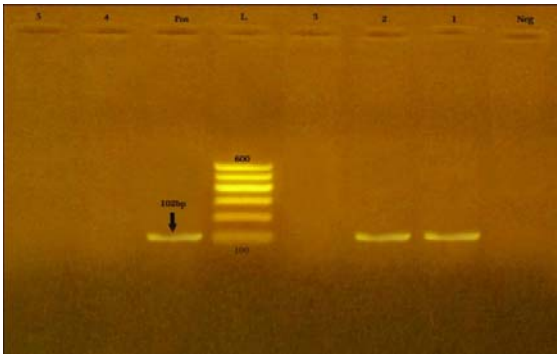

Fig. (5): Enterotoxin A (*sea)*gene. Lane L: 100-600 bp DNA Ladder. Neg.: Negative control (Listeria reference: NCINB50007). Pos.: Positive control (*S. aureus* reference: ATCC25923 at 102 bp). Lane 1& 2: *S. aureus* (Positive). Lane 3; 4&5: *S. aureus* (Negative).

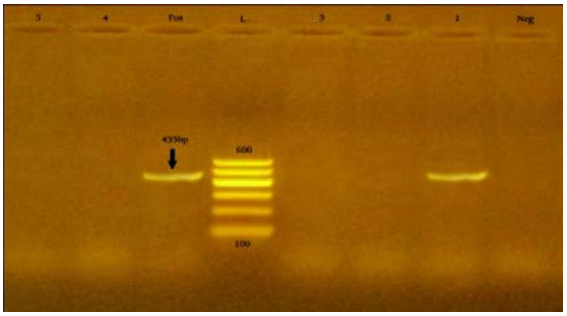

Fig. (6): Leukocidin (*pvl*) gene. Lane L: 100-600 bp DNA Ladder. Neg.: Negative control (Listeria reference: NCINB50007). Pos.: Positive control (*S. aureus* reference: ATCC25923 at 433 bp). Lane 1: *S. aureus* (Positive). Lane 2; 3; 4 & 5: *S. aureus* (Negative).

Tobramycin. These results were agreed with Al-Ghamdi (2012); Otalu et al. (2011); Suleiman et al. (2013) Abdalrahman et al. (2015) and Aklilu et al. (2016). The resistance to methicillin occurred mainly due to the presence of *mecA* gene on *S. aureus* chromosome that responsible for the production of Penicillin binding protein PBP2a. (Ito et al., 2004). In addition, the results proved that multiple antibiotic resistances are widely spread among isolated strains of *S. aureus* and decided the fact of Mathur and Singh (2005)) that the food chain can be considered as the main route of transmission of antibiotic resistant bacteria between the animal and human populations. Moreover, the isolated *S. aureus* strains were highly sensitive to Gentamycin followed by Enrofloxacin; Norfloxacin; Lomefloxacin and Ciprofloxacin. These results were agreed with Waters et al. (2011) Al-Ghamdi (2012); Suleiman et al. (2013) and Abdalrahman et al. (2015).

S. aureus is important pathogen in relation to poultry meat hygiene because of its ability to produce enterotoxins. These enterotoxins are serologically grouped into four major classical types which are SEA, SEB, SEC and SED detected by reversed passive latex agglutination kit (SET-RPLA) (Bendahou et al., 2009; Zouharova and Rysanek, 2008). The results of SET- RPLA test revealed that 5 strains out of 10 randomly examined strains (50.0%) were enterotoxigenic and classified according to type of toxin into (3A;1 B and 1C). This result nearly similar to that recorded by Gad (2004) Eshraghi et al. (2009) and Abdalrahman et al. (2015).

The PCR technique is capable of identifying the pathogenic *S. aureus* isolates and identifying the virulence factors of them (Momtaz et al., 2013). The results of PCR for amplification of *femA* gene in *S. aureus* isolates (Fig., 1) showed that, the *femA* gene was amplified in all 5 studied strains (100.0%) giving product of 132 bp. The results came in harmony with those of Pelisser et al. (2009) and Al-Khafaji and Flayyih (2015.). The results of PCR for amplification of *mecA* gene in *S. aureus* isolates (Fig., 2) showed that, the *mecA* gene was amplified in all 5 studied strains (100.0%) giving product of 310 bp. Similar detection of *mecA* gene in *S. aureus* strains (MRSA) isolated from chicken meat and its products were recorded by Bunnoeng et al. (2014); Fessler et al. (2011); Lim et al. (2010); Lozano et al. (2009); Momtaz et al. (2013); Weese et al. (2010) and Ahmed (2015).Moreover, *mecA* alone does not solely confer the methicillin resistance as it was detected among MSSA isolates and studies have shown that fem (factors essential for methicillin-resistance) or the auxiliary genes like femA/B/X in addition to mecA are important in expression of methicillin resistance, the femABX operon encodes factors which are responsible for the formation of pentaglycine bridges in the cell wall of Staphylococci (Carroll, 2008; Chikkala et al., 2012) and there was correlation between genotypic content of the *femA* and *mecA* genes and the phenotypic expression of them when tested by antibiotic disc diffusion method.

In addition, Abdalrahman et al. (2015) failed to detect *mecA* gene in poultry meat and its products and said that, this might be due to over production of β-lactamase enzymes or the presence of a variant *mecA* gene that does not amplify with the available PCR primers. Regarding to the occurrence of β-haemolysin (*hlb*) gene in *S. aureus* isolates.

The obtained result revealed that it was amplified in all 5 studied *S. aureus* strains (100.0%) giving product of 496 bp as shown in Fig. (3). These results came in accordance with those recorded by Fessler et al. (2011) and (Abdalrahman et al., 2015). The results of PCR for amplification of *icaA* gene in *S. aureus* isolates (Fig.,4) showed that, the *icaA* gene was amplified in 4(80.0%) *S. aureus* strains giving product of 1315 bp. Similar findings were recorded by Eftekhar and Dadaei (2011) and Fessler et al. (2011).

Staphylococcal enterotoxins are mostly carried on mobile genetic elements, which enable them to transfer horizontally among bacterial populations (Fitzgerald et al., 2001). The results of PCR for amplification of Enterotoxin A (*sea*) gene in *S. aureus* isolates (Fig., 5) showed that, the *sea* gene was amplified in 2 (40.0%) *S. aureus* strains giving product of 102 bp. Nearly similar results obtained by Aydin et al. (2011); Madahi et al. (2014); Moon et al. (2007); Zargar et al. (2014) and Abdalrahman et al. (2015). Meanwhile, the results were disagreed with Fessler et al. (2011) who failed to detect sea gene in S. aureus strains isolated from poultry and poultry products. The results of PCR for amplification of Panton-Valentine Leukocidin (*pvl*) gene of *S. aureus* (Fig.,6) revealed that, the *pvl* gene was amplified in one *S. aureus* strains of chicken meat sample only (20.0%) giving product of 433 bp. These results were agreed with those obtained by Abdalrahman et al. (2015); Djoudi et al. (2013). Meanwhile, the results were disagreed with Fessler et al. (2011) who failed to detect *pvl* gene in *S. aureus* MRSA strains isolated from poultry and poultry products.

Finally, the recorded results demonstrate that, *S. aureus* and MRSA strains are commonly found in retail raw chicken meat and its common products sold in markets in Kaliobia Governorate. Presence of MRSA in raw chickens may pose a potential threat to human health. Measures including good

manufacturing practice and hazard analysis and critical control points systems should be taken to control the pathogen in poultry production at the farm, processing and retail level.

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