Molecular characterization for some virulence and antibiotic resistance genes of *Staphylococcus aureus* isolated from dairy cattle's subclinical mastitis in EL- Sharkia Governorate.

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ABSTRACT

Staphylococcus aureus has emerged as a significant public health problem as it is often responsible for intramammary infection in bovine. The emergence of MRSA in animals was from an outbreak of mastitis in cattle which represent a great economic in milk industry. The present study was carried out to genotypically characterized *S. aureus* isolated from subclinical bovine mastitis in different farms in EL- Sharkia Governorate. A total of 15 *S. aureus* isolates were obtained from 100 subclinical mastitic milk samples and subjected to PCR for detection of some virulence and antimicrobial resistance genes using oligonucleotide primers that amplified genes encoding enterotoxin genes A to E (*sea, seb, sec, sed, see*), coagulase gene (*coa*), the IgG binding region of protein A (*spa*) and resistance gene as factor essential for expression of methicillin resistance (*fem*A) which was used as an internal positive control and intrinsic methicillin resistance gene (*mec*A). PCR amplification revealed that all *S. aureus* isolates were enterotoxogenic and MRSA, harbored the genes encoding staphylococcal coagulase and the genes encoding the immunoglobulin G binding region of protein A. The data in the study provided an overview on the distribution of virulence determinants of MRSA strains which contributed to bovine mastitis problem in the Egypt farm.

Keywords: *Staphylococcus aureus,* Polymorphism, Genotyping, Virulence factors, Mastitis.

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1. INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen in dairy ruminant where it is found in healthy carriage and can be a major cause of mastitis (Seyffert et al., 2012). *S.aureus* is recognized worldwide as a frequent cause of subclinical intramammary infections in dairy cows. The main reservoir of *S. aureus* seems to be the infected quarter and transmission between cows usually occurs during milking *S. aureus* produces a spectrum of extra cellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism (Momtaz et al., 2010). A variety of virulence factors that is responsible for subclinical and persistent intramammary infections (Fitzgerald et al., 2000). *Staphylococcal* enterotoxins (SEs) are serologically grouped into five major classical types. However, other new enterotoxins have been described (Thomas et al., 2007). *Sea* and *seb* are usually more common in milk and milk products (Chiang et al., 2006). Several studies reported the production of SEs or the presence of toxin genes in *Staphylococcus aureus* from milk and derivate associated with mastitis cows in different countries (Normanno et al., 2005). Additionally, coagulase has also

been shown to be a virulence factor in intrammmary infection. This protein is coded by *coa gene* that possesses a conserved and a repeated polymorphic region that can be used to measure relatedness among *S. aureus* isolates (Reinoso, 2004). Coagulase protein has the ability to turn fibrinogen into fibrin threads by a mechanism different from natural clotting (Palma et al*.,* 1999). *Staphylococcus aureus* encodes many virulence factors including the surface IgG-binding protein A (*spa*) whose function is to capture the Fc region of immunoglobulin of most mammalian species therefore prevent phagocytosis of the bacterial cells by the host immune system (Foster, 2005). Besides virulence factors, the increased resistance of *S. aureus* isolated from mastitic cows to several antimicrobial agents has been reported (Gentilini et al*.,* 2000), what impacts the effectiveness of therapy since control methods of this organism from dairy herds requires treatment of infected mammary glands with effective antimicrobial agents (Kirkanet al*.,* 2005). MRSA detection has been reported in milk from mastitic cows (Goniet al*.,* 2004). The most commonly known carrier of the *mec*A gene is the bacterium known as MRSA. *Mec*A, a structural gene located on the chromosome of *S. aureus*, characterizes methicillin resistant *S. aureus* (MRSA) and *fem*A gene encode proteins which influence the level of methicillin resistance of *S. aureus* (Kobayash et al.,1994). The outcome of this work is molecular detection of virulence genes of *S. aureus* involving in subclinical mastitic cases beside the acquition of methicillin resistance as little information is available about virulence determinant of these bacteria in Egypt.

2. MATERIAL AND METHODS

2.1. Bacterial Isolates and culture media

Milk samples were collected aseptically from 475 cows with subclinical mastitis in EL-Sharkia Governorate. Milk samples from apparently healthy animals were tested for subclinical mastitis by CMT and SCC (Radostits et al*.,* 1994) and (Kot et al*.,* 2012), respectively. Milk samples for CMT positive animals (considered as SCM positive) were induced in the present study for detection of *S. aureus*. A total of coagulase positive *Staphylococcus* isolates was included in this investigation. Milk samples were inoculated onto blood agar base (Merck) supplemented with 5% defibrinated sheep blood. Isolates were identified by conventional methods, including Gram staining, colony morphology, haemolysis tests for catalase, coagulase and anaerobic fermentation of mannitol (Konemanet al*.,* 2001). All strains were stored on suitable maintenance media in the National Laboratory for Bacteriology, Laboratory center for PCR. Bacterial cultures were grown in brain heart infusion broth prior to extraction of total DNA.

2.2. PCR amplification

PCR amplification was performed with PTC-100 programmable thermal cycler (Peltier Effect cycling, MJ, Research, INC, UK) in a volume of 50 ml consisting of: 12.5µl of Emerald Amp GT PCR master mix (2x premix), 1 µl of 20 pmol of each primer for one sample, 6 µl of the DNA template and water, nuclease-free up to 25 µl in uniplex PCR. While, 25µl of Emerald Amp GT PCR master mix (2x premix), 1 µl of 20 pmol of each primer for one sample, 10µl of the DNA template and water, nuclease-free up to 50 µl in PCR.

Primer sequence and PCR amplification cycles of oligonucleotide primers among the selected isolates are illustrated in Tables (1 and 2).

3. RESULTS

One hundred (21%) samples from 475 cows were found to be CMT positive. On the basis of cultural and biochemical properties, 61 isolates were identified as *S. aureus*. All 61 isolates were positive for the catalase test, the tube coagulase test,

hemolysis on blood agar and gave golden pigment on milk agar. The results of PCR for 15 *S. aureus* isolates revealed that all 15 isolates of *S. aureus* were enterotoxigenic, positive for coagulase gene with polymorphism and *spa* gene. The amplification of the *coa* gene displayed three different size polymorphisms with about 430bp, 630bp and 750bp as shown in fig. (1). *Spa* gene represented IgG binding region of protein A with revealing size of 226 bp (Fig. 2). Additionally, all examined isolates was enterotoxiginic with predominance for *see* in uniplex. Thirteen (86.7%) isolates were positive for enterotoxin A gene, 6 (40%) isolates harboured enterotoxin B gene and enterotoxin C gene. Inionically, *sed* was not detected in any *S. aureus* isolates as shown in fig. (5, 6, 7, 8 and 9). All 15 isolates were positive for *fem*A and *mec*A genes yielding an amplicon size of 132bp and 310bp, respectively (Fig. 3 and 4). The results of multiplex PCR for 15 *S. aureus* showed that the predominant enterotoxigenic gene was *see* gene followed *sea* and *seb* among *S. aureus* isolates. Additionally, it was also found in combination with *sea* and *seb* genes as shown in fig. (10).

Table (1): Oligonucleotide primers sequences of all primers used in PCR amplification assays and their respective PCR product size

Gene	Primer	Primer sequence (5'-3')	Length of amplified product
sea	SEA-F	GGTTATCAATGTGCGGGTGG	102 bp
	SEA-R	CGGCACTTTTTTCTCTTCGG	
seb	SEB-F	GTATGGTGGTGTAACTGAGC	164 bp
	SEB-R	CCAAATAGTGACGAGTTAGG	
sec	SEC-F	AGATGAAGTAGTTGATGTGTATGG	451 bp
	SEC-R	CACACTTTTAGAATCAACCG	
sed	SED-F	CCAATAATAGGAGAAAATAAAAG	278 bp
	SED-R	ATTGGTATTTTTTTTCGTTC	
see	SEE-F	AGGTTTTTTCACAGGTCATCC	209 bp
	SEE-R	CTTTTTTTTCTTCGGTCAATC	
mec	*mec*A-1	GTAGAAATGACTGAACGTCCGATAA	310 bp
A	*mec*A-2	CCAATTCCACATTGTTTCGGTCTAA	
coa	Coa-F	ATAGAGATGCTGGTACAGG	Four different types of bands may be detected
	Coa-R	GCTTCCGATTGTTCGATGC	
			350 bp
			430 bp
			570 bp
			630 bp
spa	Spa-F5	TCA ACA AAG AAC AAC AAA ATG C	226 bp
	Spa-R8	GCT TTC GGT GCT TGA GAT TC	
*fem*A	FEMA-F	AAAAAAGCACATAACAAGCG	132 bp
	FEMA-R	GATAAAGAAGAAACCAGCAG	

Table (2): Cycling conditions for amplification of some virulence and resistant genes, among *S. aureus* isolates.

Target gene	Initial denaturation	Actual cycles	Final extension	Reference
sea, seb and *see*	94˚C 5 min.	35 cycles of : Denaturation: 94˚C/30 sec. Annealing: 50/30 sec. Extension: 72/30 sec.	72˚C 7 min.	Mehrotra et al., 2000
sec	94˚C 10 min.	35 cycles of : Denaturation: 94˚C/45 sec. Annealing: 50/45 sec. Extension: 72/45 sec.	72˚C 10 min.	
sed	94˚C 5 min.	35 cycles of : Denaturation: 94˚C/30 sec. Annealing: 48/30 sec. Extension: 72/30 sec.	72˚C 7 min.	
*mec*A	94˚C 10 min.	35 cycles of : Denaturation: 94˚C/45 sec. Annealing: 50/45 sec. Extension: 72/45 sec.	72˚C 10 min.	McClure et al., 2006
coa	94˚C 10 min.	35 cycles of : Denaturation: 94˚C/1 min. Annealing: 55/1 min. Extension: 72/1 min.	72˚C 10 min.	Iyer and Kumosani, 2011
spa	94˚C 5 min.	35 cycles of : Denaturation: 94˚C/30 sec. Annealing: 55/30 sec. Extension: 72/30 sec.	72˚C 7 min.	Wada et al., 2010
*fem*A	94˚ 5 min.	35 cycles of : Denaturation: 94˚C/30 sec. Annealing: 50/30 sec. Extension: 72/30 sec.	72˚C 7 min.	Mehrotra et al., 2000

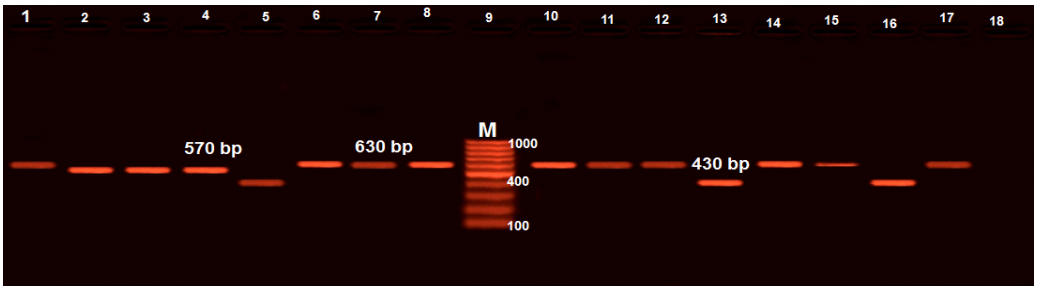

Fig. (1): Agarose gel electrophoresis of PCR products after amplification of *coa* gene with amplified product at 430 bp, 570 and 630bp. Lane (9) (M):100-600bp DNA Ladder "Marker" (100 Pharmacia). Lanes (5, 13 and16): positive carrying coagulase gene at 430 bp. Lanes (2, 3 and4): positive carrying coagulase gene at 570 bp. Lanes (1, 6, 7, 10, 11, 12, 14, 15 and 17): positive carrying coagulase gene at 630bp. Lane (8): Positive control (reference strain deposited to gene bank with accession no.Z33404). Lane (18): Negative control.

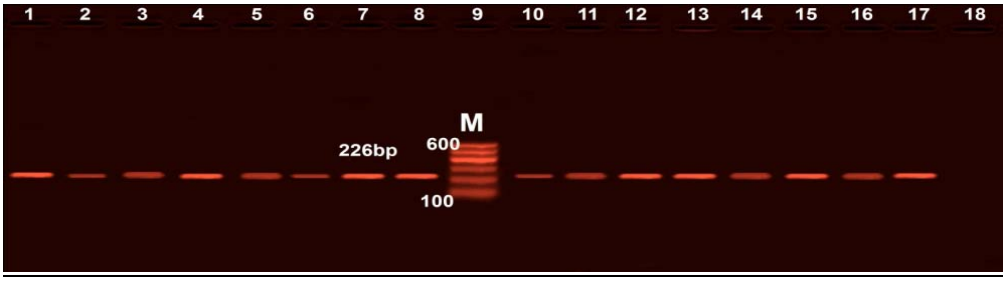

Fig. (2): Agarose gel electrophoresis of PCR products after amplification of *spa* (IgG-binding protein) gene at 226 bp amplified product. Lane 9 (M): 100-600bp DNA Ladder "Marker" (100 Pharmacia). All lanes (1: 17): positive isolates at 226 bp. Lane 8: Positive control (reference strain deposited to gene bank with accession no. P38507). Lane 18: Negative control.

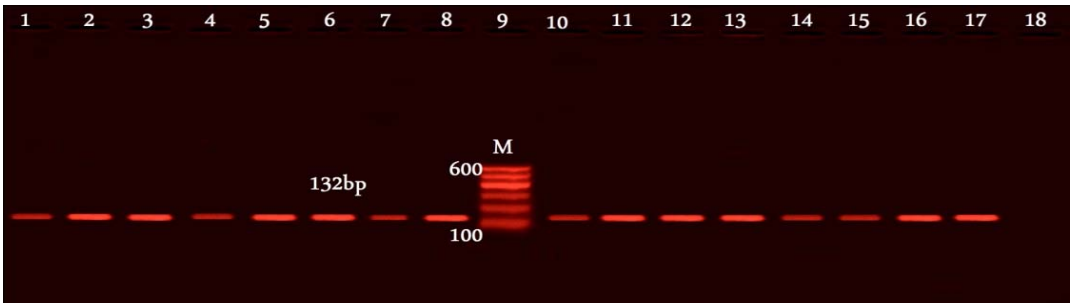

Fig. (3): Agarose gel electrophoresis of PCR products after amplification of *fem*A gene at 132 bp amplified product. Lane 9 (M): 100-600bp DNA Ladder "Marker" (100 Pharmacia). All lanes (1:17): positive isolates at 132 bp. Lane 8: Positive control (reference strain deposited to gene bank with accession no.NC_002952). Lane 18: Negative control.

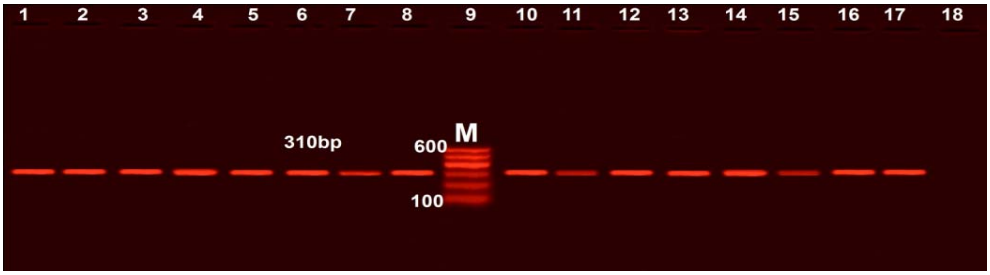

Fig. (4): Agarose gel electrophoresis of PCR products after amplification of *mec*A gene at 310bp amplified product. Lane 9 (M): 100-600bp DNA Ladder "Marker" (100 Pharmacia). All lanes (1:17): positive isolates at 310 bp. Lane 8: Positive control (reference strain deposited to gene bank with accession no.X52593). Lane 18: Negative control.

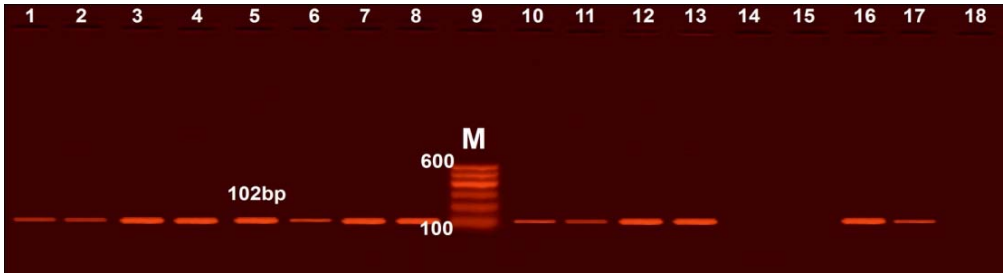

Fig. (5): Agarose gel electrophoresis of PCR products after amplification of *sea* gene at 102bp amplified product. Lane 9 (M): 100-600bp DNA Ladder "Marker" (100 Pharmacia). Lanes (1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 16 and 17): positive isolates at 102 bp. Lanes (14and 15): negative isolates. Lane 8: Positive control (reference strain deposited to gene bank with accession no. DQ641635). Lane 18: Negative control.

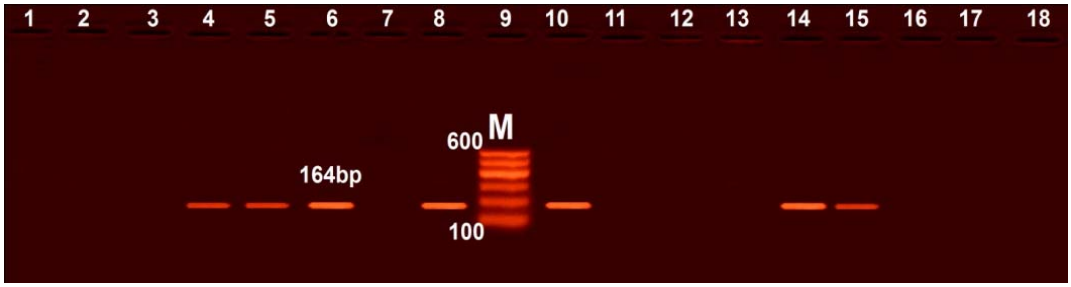

Fig. (6): Agarose gel electrophoresis of PCR products after amplification of *seb* gene at 164 bp amplified product.Lane 9 (M): 100-600bp DNA Ladder "Marker" (100 Pharmacia). Lanes (4, 5, 6, 10, 14, 15): positive isolates at 164 bp. Lanes (1, 2, 3, 7, 11, 12, 13, 16, 17): negative isolates. Lane 8: Positive control (reference strain deposited to gene bank with accession no.AY518386). Lane 18: Negative control.

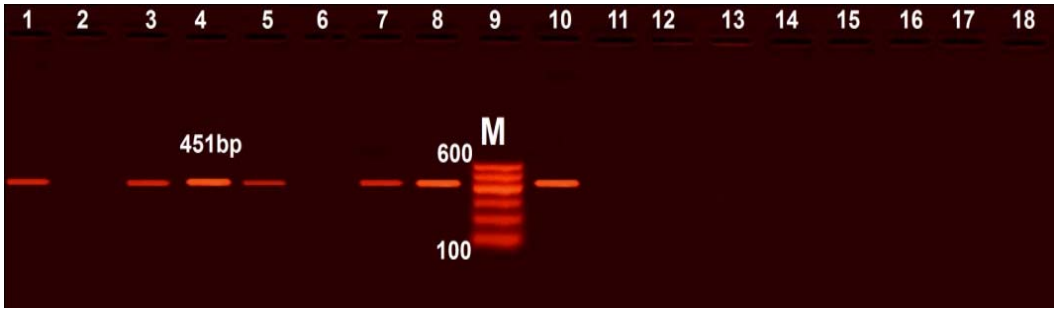

Fig. (7): Agarose gel electrophoresis of PCR products after amplification of *sec* gene at 451bp amplified product. Lane 9 (M): 100-600bp DNA Ladder "Marker (100 Pharmacia). Lanes (1, 3, 4, 5, 7 and 10): positive isolates at 451 bp. Lanes (2, 6, 11, 12, 13, 14, 15, 16 and 17): negative isolates. Lane 8: Positive control (reference strain deposited to gene bank with accession no.AB084256). Lane 18: Negative control.

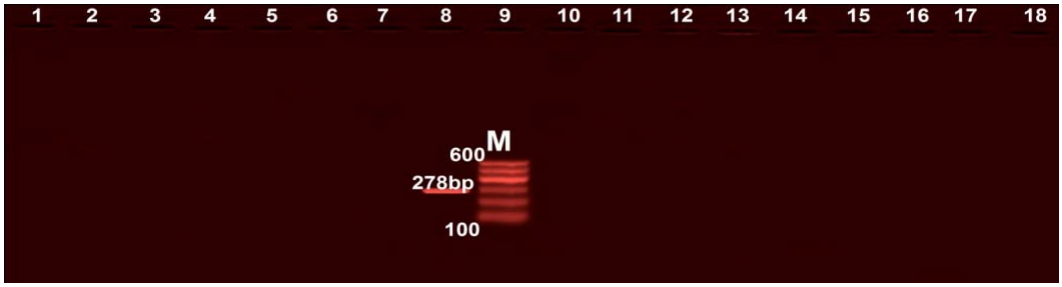

Fig. **(8):** Agarose gel electrophoresis of PCR products after amplification of *sed* gene at 278 bp amplified product. Lane 9 (M): 100-600bp DNA Ladder "Marker (100 Pharmacia). All lanes (1:17): negative isolates. Lane 8: Positive control (reference strain deposited to gene bank with accession no.AY518388 at 278 bp).Lane 18: Negative control.

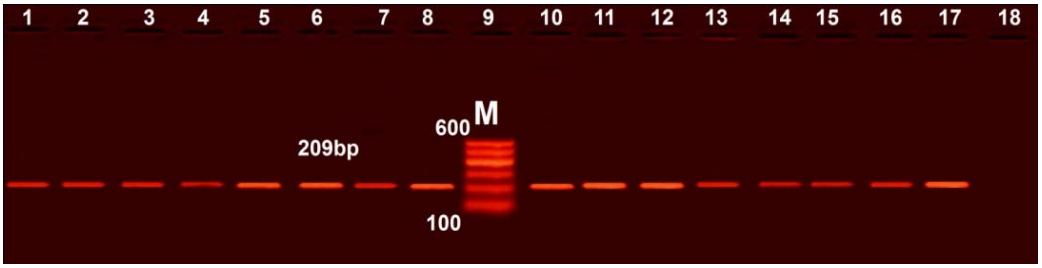

Fig. (9): Agarose gel electrophoresis of PCR products after amplification of *see* gene at 209 bp amplified product. Lane 9 (M): 100-600 bp DNA ladder "Marker (100 Pharmacia). All Lanes (1:17): positive isolates at 209 bp. Lane 8: positive control (reference strain deposited to gene bank with accession no.AY518387). Lane 18: Negative control.

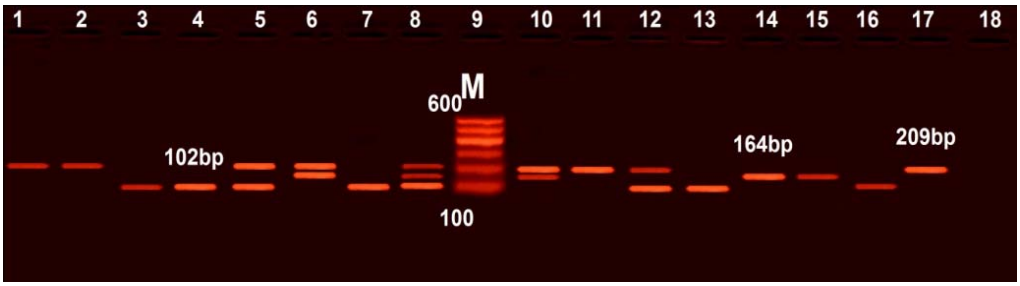

Fig. (10): Agarose gel electrophoresis of PCR products after amplification of *sea, seb* and *see* virulence genes in a multiplex PCR. Lane 9 (M):100-600 bp DNA Ladder "Marker" (100 Pharmacia). Lanes (1, 2,11and17): *S. aureus* had one virulence gene *see* only at 209 bp. Lanes (3, 4, 7, 13 and 16): *S. aureus* had one virulence genes *sea* only at 102 bp. Lanes (14 and 15): *S. aureus* had one virulence genes *seb* at 164 bp. Lanes (5 and12): *S. aureus* had both *sea* and *see* virulence genes. Lanes (6 and10): *S. aureus* had both *seb* and *see* virulence genes. Lane 8: Positive control (reference strain). Lane 18: Negative control.

4. DISCUSSION

S. aureus is recognized worldwide as a frequent cause of subclinical intramammary infections in dairy cows (Straub et al., 1999). The identification of 15 *S. aureus* isolates in the present study could be performed by conventional methods and by PCR technology using nucleotide primers targeting different gene. Various virulence genes encoding coagulase gene, the IgG binding region of protein A and enterotoxins (*sea-see*). Comparable PCR base detection studies have been described

by other investigators (Algammal et al., 2013).

The amplification of the *coa* gene displayed three different size polymorphisms with approximately 430 bp for 3 isolates (20%), 570 bp for 3 isolates (20%) and 630 bp for 9 isolates (60%). This attributed to the presence of more than one allelic form of the coagulase gene as mentioned by Aslantas et al., (2007). These results are in exact with the findings of Gharib et al., (2013) who suggested that an amplicon of about 600 bp are predominant in bovine strains. On the other hand, Schledgelova et al., (2003) reported the size of *coa* gene PCR product of *S. aureus* isolates from dairy cow gave 4 classes at 650, 730, 810 and 1050 bp. Class 730 bp was the most common class between the isolates.

The other virulence factor with great concern is staphylococcal enterotoxins as they are very resistant to heat and gastrointestinal protease that justifies why they remains active after thermal processing and the genes responsible for encoding these enterotoxins were detected by uniplex and multiplex PCR. Production of *sea, seb, sec, sed*, and *see* by *S. aureus* strains associated with bovine mastitis has been described by Rall et al., 2008.

Uniplex and multiplex PCR results obtained in this study showed that 100% of *S. aureus* isolates were positive for one or more enterotoxin. The occurrence of multiple toxin genes in *S. aureus* was considered rare (Jorgensen et al., 2005). This supports the suggestion that SE and SE-like toxins may cause bovine mastitis by depressing the bovine immune system (Ferens et al., 1998).

Among these classical SE genes *see* and *sea* showed the highest prevalence rate in the present study. This is a cause of concern as a potential health risk for humans, because most *S. aureus* strains that possess the *see* and *sea* gene produce toxins, which is a major etiological factor of staphylococcal food poisoning (Hwang et al., 2010).

The predominant classical SE varied from country to country: In Tehran& Mashhad see gene (Sahebekhtiari et al., 2011); In Canada, see and sea gene (Mehrotra et al., 2000); In Brazil, *sea* gene (Rall et al., 2014); In Hungary, *seb* gene (Zouharova and Rysanek, 2008); In Iraq and Argentina, *sec* gene (Khudor et al., 2012) and (Nederet al., 2011), respectively; In Italy, *sed* gene (Carfora et al., 2015) and here in Egypt *see* was found in 100% (15/15) by uniplex PCR. Additionally, it was also found in combination with *sea* and *seb* genes by multiplex PCR. The prevalence of *see* gene and combination with other enterotoxins has reported elsewhere (Mehrotra et al., 2000) and (El-Seedy et al., 2010). Irionically, in this study *sed* gene is not detected neither by uniplex nor multiplex PCR. It was matched with Abd EL-Tawab, et al., (2015).

It is worth mentioning that, Protein A is a component of *S. aureus* cell wall and is covalently bound to the peptidoglycan. The PCR amplification of the gene encoding the IgG-binding region of protein A revealed band of 226 bp for all *S. aureus* strains (100%), These results agreed with those obtained by Enany et al., 2013. While other authors detected the immunoglobulin G binding region of protein A with different percentage as (Mehndiratta, et al., 2009) (94.6%) and (Bekhit et al., 2010) (32.4%).

In addition to virulence, a major concern in the control of mastitis is resistance of the etiological agent to antibiotics. Staphylococcal resistance to methicillin is increased nowadays and associated with acquisition of the staphylococcal cassette chromosome *mec*A and *fem*A which is a protein encode the level expression of methicillin resistance (kumaret al., 2010). Therefore, the detection of *fem*A and together with *mec*A by PCR was considered to be a more reliable indicator to identify MRSA (Vannuffel et al., 2000).

The *fem*A gene product, a 48-kDa protein (cytoplasmic protein), has been suggested to have a role in cell wall metabolism and is reported to be present in all *S. aureus* species during the active growth phase. This gene was a necessary for the expression of

methicillin resistance in *Staphylococcus aureus* and also involved in the biosynthesis of staphylococcal cell walls (Johnson et al*.*, 1995) and is universally presented in all *S. aureus* isolates (100%) similar as Mehrotra et al*.*, (2000).

In the current study genotypically *mec*A gene was detected with high percent (100%) in all *S. aureus* isolates with a 310 bp amplified product matched with Omar et al*.*, (2014) in Egypt; Mehrotra et al*.*, (2000) in Canada and Saidi et al*.*, (2015) in Algeria. This high rate of methicillin resistance can be attributed to the random use of methicillin in farms of delta region and the incidence of *mec*A gene represent a great hazard to public health as it may be transfer to human.

5. CONCLUSION

Data presented in this study showed abroad distribution of identical related *S. aureus* clones are responsible for the mastitis situations in Egypt with highly prevalence rate of methicillin resistance among the obtained isolates which represent an alarm for a great hazard to public health.

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