

Bacteriological and molecular studies on Staphylococcus aureus isolated from mastitic cowsAbd El Hamid, T. M¹, Sahar, R. Mohamed², and I. A. Saleh³¹Veterinary Hospital, Faculty of Veterinary Medicine, Cairo University, Egypt²Animal Health Research Institute (Dokki branch), Egypt³Faculty of Veterinary Medicine, Cairo University, Egypttaherelrefaey@yahoo.com

Abstract: Inflammation of mammary gland in bovines is frequently due to infection by *Staphylococcus aureus* which leads to appearance of clinical and subclinical mastitis in cattle. In this research the mastitic cases (103) were classified into clinical and subclinical cases (47,56) respectively. All milk samples collected from infected cows were subjected to bacteriological examination and molecular characterization of some *Staphylococcus aureus* isolates. *Staphylococcus aureus* was isolated from clinical and subclinical mastitic cows in an incidence of (50%, 17.5%) respectively. The application of multiplex PCR on some *Staph. aureus* isolates (8) was effectively in detection of, *tst*, *hlg*, *clfA*, *nuc* genes by amplification at a single amplicon at (326bp, 937bp, 638bp, 395bp respectively).

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Introduction

Milk is one of the main sources for proteins, fats and minerals and considered as a complete diet for infants and younger ages. Despite, milk is a favorable media for growth of several types of bacteria. There are several sources sharing in contamination of milk with microorganisms such as milking personnel (unclean hands, udder polluted with mud etc.), utensils for collection of milk and the manipulation method with milk during lactation and transfer or storage.

Mastitis is recognized as the most important dairy herd problems worldwide. Economic losses of mastitis include decrease in milk quantity and quality and high cost treatment. *S.aureus* is one of the most common etiological pathogens, causing intramammary infections in lactating animals which result in drop in milk yield, deterioration of milk and causing serve losses in the income in breeders around the world (OldeRiekerink et al., 2010).

One of the principle causes of mastitis in cattle is *S. aureus* infection, where it settled in the infected quarter and can transmitted to the remaining quarters or between cows via milking processes. The pathogenesis of mastitis may be attributed to the secretion of cytotoxins (hemolysin and leukotoxin) by *S. aureus* which responsible for the pathogenesis of mastitis in animals. Some studies reported that hemolysin genes and leukocidin were responsible for *Staphylococcus* in mastitic milk (Jahan et al., 2015). The ability of *S.aureus* to secrete many types of extra cellular toxicants in addition to several virulent factors may be play an important role in the pathogenesis of

S.aureus; while TST-1 was found to be a cause of toxic shock symptoms because it contain super-antigenic exotoxin (Fueyo et al., 2005). Some researchers reported that *S. aureus* have the capability to stick on extracellular matrix proteins which may be necessary for the colonization and the occurrence of infections (Salasia et al., 2004). In addition, El-Sayed et al., 2005 found that *S.aureus* carry different adhesion genes such as *clfA*, *fnbA*. Application of PCR technique for examination of 8 strains of *S.aureus*, the results revealed to the presence of *clfA* genes in the examined samples (Fig.4), they suggested its role in the pathogenicity of udder inflammation in bovines.

There are many factors are may be responsible for the pathogenesis of mastitis such as extracellular toxins, surface antigens and enzymes (O’Riordan and Lee, 2004).

The presumptive *S. aureus* isolates were confirmed by amplifying species –specific thermonuclease (*nuc*) gene (Hamid et al., 2017).

Therefore, the present work aimed to this study role and incidence of *Staphylococcus aureus* infection in cows suffering from subclinical and clinical mastitis, also, to study the genotypic categorization of some strain of *S. aureus* and their virulence which isolated from milk of mastitic cows and can supply with a data about distribution of virulence determinants of these *S. aureus* strains which share in induction of bovine mastitis troubles in the Egyptian farms.

Material & methods

1-Samples

A total of 412 milk samples were collected from clinically and sub clinically infected cases the samples were transferred in ice box directly with an hour to the laboratory with a minimum delay to be bacteriologically examined (Quinn et al., 2002).

2-Bacteriological examination

All samples were inoculated onto blood agar base (Merck) supplemented with 5% defibrinated sheep blood and mannitol salt agar plates and incubated aerobically at 37°C for 24h Suspected colonies were picked up for purification and subjected for identification microscopically and biochemically according to (Colle et al., 1996), Quinn et al., 2002, 2003 and Freitas et al., 2013) isolates were identified

by conventional methods, including Gram staining, colony morphology, hemolysis test, catalase, coagulase and anaerobic fermentation of mannitol (Konman et al., 1992).

3-Detection of virulence genes of Staphylococcus aureus by PCR

Primers for detection of 4 virulence gene of Staphylococcus aureus, these genes were, hlg, tst, nuc and clfA. It was applied on 8 random isolates of Staphylococcus aureus following QIAamp® DNA Mini kit instructions (catalogue no. M501DP100), Emerald Amp GT PCR mastermix (Takara) with code NO. Rr310A and agarose gel electrophoresis (Sambrook et al., 1989) sequence of primer used are illustrated in Table 1.

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

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
tst	F-ACCCCTGTTCCTTATCATC	326 bp	Farahmand et al., 2013
	R-TTTCAGTATTTGTAACGCC		
hlg	F-GCCAATCCGTTATTAGAAAATGC	937 bp	Kumar et al., 2009
	R-CCATAGACGTAGCAACGGAT		
clfA	F-GCAAAATCCAGCACAAACAGGAAACGA	638 bp	Scherrer et al., 2004
	R-CTTGATCTCCAGCCATAATTGGTGG		
nuc	F-ATATGTATGGCAATCGTTTCAAT	395 bp	Morandi et al., 2010
	R-GTAAATGCACTTGCTTCAGGAC		

Cycling conditions of the primers during PCR

Table (2): Temperature and time conditions of the primers during PCR are shown in

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
tst	94°C 10 min.	94°C 45 sec.	50°C 45 sec.	72°C 45 sec.	35	72°C 10 min.
hlg	94°C 10 min.	94°C 1 min.	55°C 1 min.	72°C 1 min.	35	72°C 10 min.
clfA	94°C 10 min.	94°C 45 sec.	55°C 1 min.	72°C 1 min.	35	72°C 10 min.
nuc	94°C 10 min.	94°C 45 sec.	55°C 45 sec.	72°C 45 sec.	35	72°C 10 min.

Results

Detection of virulence genes of S.aureus isolated from mastitic cattle

nuc gene (Thermonuclease) amplified at 395bp were 3 positive and 5 negative in an incidence of (37.5%) and (62.5%) respectively also tst gene (Toxic shock syndrome gene) amplified at 326pb were 2 positive and 6 negative as (25%) and (75%)

respectively as shown in Fig (1), clfA gene (clumping factor) amplified at 638bp where 3 were positive 5 negative in an incidence of (37.7%) and (62.5%) respectively as shown in (Fig 2) also hlg gene hemolysin gene amplified at 937 bp where 4 were positive and 4 negative hlg gene positive were (50%) and negative were (50%).

Table (3): incidence of Staphylococcus aureus in the mastitic cows

Animal case	No	No of quarter	S. aureus isolates	
			No	%
Clinically mastitic	47	188	94	50
Subclinical mastitic	56	224	39	17.5
Total	103	412	133	30.9

% calculated according to the no. of quarter examined

S. aureus was isolated from 94 out of 188 and from 39 out of 224 collected from clinically and subclinically mastitic cows in milk samples. Incidence of 50% and 17.5% respectively with a total incidence of 30.9%.

Percentage were calculated according to the no. of tested *S. aureus* isolates

Table (4) The results of PCR amplification of tested *S. aureus* isolates (8):

Tested genes	positive		Negative	
	No	%	No	%
<i>nuc</i>	3	37.5	5	62.5
<i>tst</i>	2	25	6	75
<i>hlg</i>	4	50	4	50
<i>clfA</i>	3	37.5	5	62.5

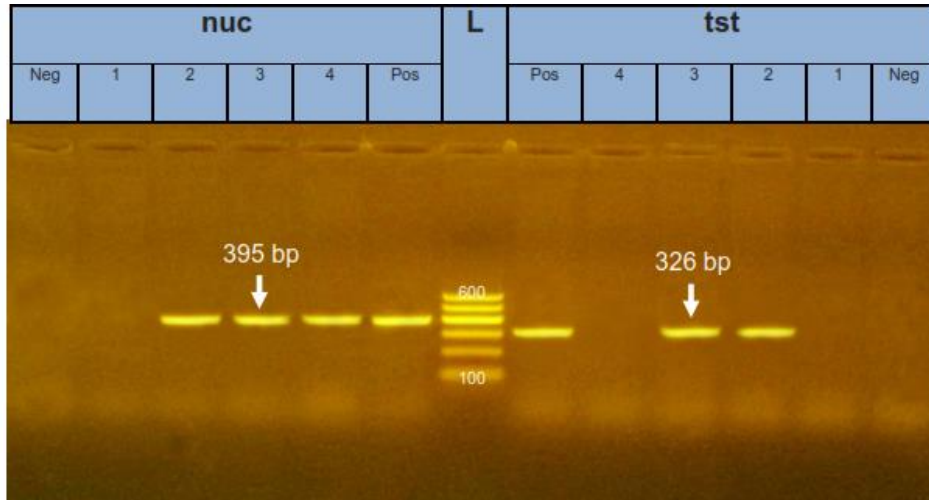


Fig (1): *nuc* gene. Lane M: 100-600 pb DNA ladder. Neg: Negative control. Pos: positive control 630bp. Lane: 2,3,4, pos. Lane: 1 Neg. *tst* gene Lane M: 100-600 pb DNA ladder. Neg: Negative control. Pos: positive control 326pb. Lane: 2,3 pos. Lane: 1,4 Neg.

The results is for (4 isolates out of 8 tested).

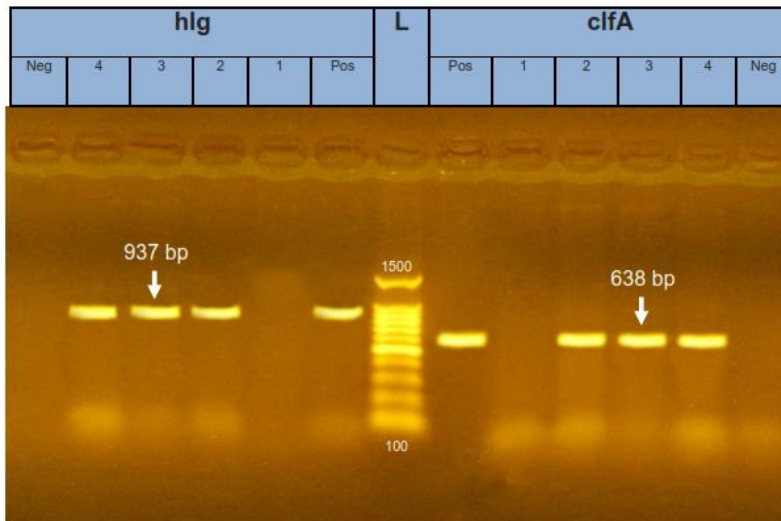


Fig. (2) *clfA* gene. Lane M: 100-1500 pb DNA ladder. Neg: Negative control. Pos: positive control 638bp. Lane: 2,3,4 pos. Lane: 1 Neg. *hlg* gene. Lane M: 100-1500 pb DNA ladder. Neg: Negative control. Pos: positive control 937bp. Lane: 2,3,4 8 pos. Lane: 1 Neg.

Discussion

Staphylococcus aureus is the most important bacterial microorganism in bovines causing contagious mastitis and highly economic losses in dairy herds.

In the present study bacteriological examination and identification of *Staphylococcus aureus* were depend on gram stain, culturing on Baird parker medium, catalase test, Coagulase tube test and DNase test.

In the present work, by using the traditional method for culturing of *Staphylococcus* on nutrient agar, different water insoluble pigments ranged between white, yellow or orange was formed on the surface of dishes. While culturing on mannitol salt agar, yields a colonies of yellow or golden yellow water insoluble pigments. Also by chemical analysis they found that *Staphylococcus* was facultative, fermented a number of carbohydrates to acid, aerobic and liquefied gelatin. The present finding are coordinated with **Jahan et al. (2015)** results.

In the present work, as shown in table 3, the incidence of *Staphylococcus aureus* infection in mastitic cows either clinical or subclinical are tabulated. The results revealed that the overall incidence of *Staphylococcus aureus* in clinical mastitis was 94% isolates out of 188, whereas it averaged 39% out of 224 in sub clinical mastitis. Concerning, clinical mastitis the incidence of *Staphylococcus aureus* was 50.0% which was higher than that recorded in sub clinical mastitis (17.50%). Overa all, the incidences of *Staphylococcus aureus* in both clinical and sub-clinical mastitis were higher. These findings are similar to previous study carried out during 2010 in the district, which stated that the microorganism *S. aureus* was responsible for inducing mastitis in dairy cattle in Mathura and its neighboring area. **Kumar et al., 2010a**, found that, the incidence of *S. aureus* in clinical and sub-clinical mastitic cattle was averaged 37.03% and 31.70%, respectively. They concluded that *S. aureus* microorganism is more prevalent in mastitic cases in dairy cattle. A different studies have been carried out in many districts in Egypt, which pointed to the prevalence of *S. aureus* as a main pathogen responsible for inducing of mastitis in dairy cattle, which agree with the present data.

Presence of *clfA* and *hla* gene (Fig.2) and protein A considered as the *Staphylococcus* species.

This agreed with **(Bedane et al., 2012)** they revealed that *S.aureus* is responsible for about 30% to 40% of all mastitis cases. This high prevalence of *S.aureus* in this study may be explained that transmission of infection occurs during the milking process by milker's hands, contaminated equipments and milking machine **(Scherrer et al., 2004)**.

Staphylococcus aureus can convey a large group of probable virulence factors such as surface proteins (that help in adherence to injured tissue); exotoxins and enzymes which is a source of a different types of infections in both skin and soft tissues, specially inflammation of mammary glands. The degree in which the microorganism can induce the disease (mastitis) depends mainly on its capability for producing extracellular toxins, surface antigens and enzymes under different environmental situation which are involoed in the pathogenesis in bovine, Three type of haemolysin α,β in the order of their discovery has been found to be produced by *S. aureus*. Alpha toxin the major cytotoxic agent of *S. aureus* was the first bacterial exotoxin to be identified as pore former which binds on a variety of cells, beta toxin has property of damaging bovine mammary secretary epithelial cell by increasing the damaging effect of α toxin, increased attachment of *S.aureus* to epithelial cells in the mammary gland and production of *S. aureus*. These finding are agreement to those of (8) it was deduced from the data obtained in the present investigation that the isolates which produced toxin in higher concentration and complete haemolysis on blood agar. This effect could have been due to synergistic effect causing complete lysis of erythrocytes used in the blood agar **(Banaiardalan et al., 2017)**.

Amplification of clumping factor A (*clfA*) gene in the present work revealed to the presence of a single amplicon with a size of approximately 638bp for all eight examined strains of *S. aureus*, as an indicator for non-occurring of polymorphisms of (*clfA*) gene.

Staphylococcus aureus is documented in the world as the common source of inducing mastitis in dairy breeds of cattle. Also, they recorded that the infected quarters of udder is the main source of occurrence of infection and the methods or procedures of milking is responsible for transmission to another lactating animals and spread of infection in the herd and surroundings. On the other hand, **Mounir et al., 2010** found that *S. aureus* secrete a large numbers of extracellular protein toxins and virulence factors which are thought to play a role in the pathogenicity of the organism.

However, **Brody et al. (2008)** found that some genes like *S. aureus* and *clfA* were found in isolates of antimicrobial resistant and susceptible strains. Statistically, the results revealed that there is a strong relationship with resistance patterns.

Amplification of genes encoding clumping factor (*clfA*) and the mononuclease (*nuc*) gene by polymerase chain reaction was used for the genotypic characterization of isolated *S. aureus* strains.

Amplification of the clumping factor (clfA) gene resulted in a single amplicon with a size of approximately 638 bp for all 8 tested *S. aureus* strains isolated from raw milk samples indicating no size polymorphisms of this gene. While the amplification of the nuc gene (Fig.1). Produced an amplicon of 395 bp in all 8 examined *S. aureus* isolated from raw milk samples. Specificity of the PCR products was demonstrated with 100% of the tested isolates. This specificity of *S. aureus* was agreed to the results recorded by **Costa et al. (2012)** and **Mohammed et al. (2015)**.

However nuc gene negative isolates and showed other isolates positive for *S. aureus*. Hence it recommended that for molecular identification of *S. aureus* nuc gene specific pcr should be carried out and amplified in the cases of all isolates that were found isolates resistant penicillin, Erythromycin, Amoxicillin and on other hand the isolates were sensitive to oxacillin and cloxacillin.

Conclusions

We can conclude from the present results that a wide sharing of identical or closely related *S. aureus* clone isolates are the main cause for induction of mastitis among lactating cows in Egypt.

The *S. aureus* isolates from mastitic cows were established to be different in distribution of their genes, where, the genotypic characterization helping in good understanding of the distribution of *S. aureus* clones between cattle mastitis isolates. This can assist in the survey and control of *S. aureus* infections in dairy cattle. More studies are required to collect much of data covering different species of animals and different areas in Egypt.

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