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STAPHYLOCOCCI AS AN ETIOLOGICAL AGENT OF SUBCLINICAL MASTITIS

By

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ABSTRACT

Subclinical mastitis is considered one of the most costly diseases in dairy production throughout the world; therefore, more attention has been focused on diagnosis of subclinical mastitis. This study was conducted to establish the prevalence of subclinical mastitis (SCM) in two dairy herds located in Ismailia and El-Sharakia Governorates with isolation of staphylococci as a causative agent of subclinical mastitis. About 230 quarter milk samples (QMS) were collected from apparently healthy hundred cows and subjected to California Mastitis Test (CMT), the positive samples were examined by bacterial culture and PCR to identify *staphylococcus aureus* as a causative agent of subclinical mastitis. The results showed that 43.5 % of the examined QMS had subclinical mastitis, all positive CMT samples were contaminated with staphylococci, biochemical identification of staphylococcal isolates revealed that 68.05 % and 21.3 % of the examined isolates were *S. aureus* and coagulase negative staphylococci(CNS), respectively. By using PCR technique, gene encoded for *S. aureus* was confirmed in 4.35 % of the examined isolates.

Keywords:

Subclinical Mastitis, CMT, PCR, CNS and *S. aureus*.

INTRODUCTION

Mastitis is an inflammation of mammary gland parenchyma which characterized by a range of physical and chemical changes in milk and pathological changes in the udder tissues **(Radostits *et al.,* 2000).** Mastitis is the most costly disease in dairy production throughout the world due to its high incidence and losses,which caused by clinical mastitis including reduced milk yield and quality, costs of veterinary care, discarded milk and shortening of productive life **(Nielsen *et al.* 2010 and Hogeveen *et al.* 2011).** However, **(Seegers *et al,* 2003 and Huijps *et al*. 2008)** found that the majority of the economic losses. The cause was subclinical mastitis that was 15 to 40 times more prevalent than the clinical form of mastitis.

Sub-clinically infected udder quarters were incriminated in developing clinical mastitis and the rate of new infections was high **(Zdunczyk *et al*., 2003).** Cows with subclinical mastitis maintain a reservoir of infection within the dairy herd and increase the potential exposure of uninfected cows to contagious pathogens **(Hortet and Seegers 1998).** Somatic cells are part of the natural defense mechanism to the inflammatory reaction of mastitis and include lymphocytes, macrophages, polymorph nuclear cells and some epithelial cells **(Pillai *et al.,* 2001)**, it can be measured quantitatively by California mastitis test (CMT), which is simple, easy and low cost screening test for subclinical mastitis at dairy farms **(Dingwell *et al.,*2003).** Most of the previous studies were directed to identify the microorganisms causing subclinical mastitis in order to establish specific and efficient management of dairy flocks to avoid the development of clinical mastitis **(McDougall *et al*., 2002 and Cheng *et al*., 2010).**

Bacteria that commonly cause mastitis are generally classified as either 'contagious' or 'environmental'pathogens,depending on the source of the pathogen and mode of transmission. *Staphylococcus aureus* is considered as typical contagious pathogens, as it is adapted to survive within the mammary gland and it was transmitted from cow to another at the time of milking **(Bradley, 2002).** *S. aureus* is a major etiological pathogen of bovine mastitis, which triggers significant economic losses in dairy herds worldwide, as it is characterized by persistent and contagious nature. *S. aureus* produces a variety of virulent factors that are responsible for subclinical and persistent intra-mammary infections. Some strains of *S. aureus* demonstrate antibiotic resistance and may persist for long time without over symptoms **(Fitzgerald *et al.,* 2000, Aires *et al.,* 2007 and Kahila *et al.,* 2015)**.

The present study aimed to detect subclinical mastitis in the bovine quarter milk samples with the prevalence of *S. aureus* and CNS as and an etiological agent of subclinical mastitis.

MATERIAL AND METHOD

1-Collection of samples according to **(APHA, 2004):**

This study was carried on two dairy herds that were located in Ismailia and El-Sharakia Governorates. About 230 quarter milk samples (QMS) were collected from 100 apparently healthy cows. Collected samples were directly transferred to the laboratory in an insulating icebox to be examined immediately**.**

2- California Mastitis Test (CMT): according to **(APHA, 2004).**

The milk positive samples to CMT were subjected to culture examination for isolation of

S. aureus. Loopful of milk samples were inoculated into nutrient broth and incubated at 37° C for 24 hours aerobically.

3- Isolation of staphylococci according to (APHA, 2004).

4- Identification of the isolated *S. aureus* organisms:

4.1. Microscopical examination according to (Murray *et al.,* 2006).

4.2. Biochemical identification according to (Quinn *et al.,* 2002).

4.3. Molecular identification of *S. aureus* by polymerase chain reaction (PCR) according to **(Mason *et al.,* 2001):**

4.3.1. Extraction of DNA (using QIAamp DNA mini kit)

4.3.2. Preparation of PCR Master Mix according to **Emerald Amp GT PCR master mix (Takara)** Code No.**RR310A** kit, using primer sequence of:

*S. aureusclfA*GCAAAATCCAGCACAACAGGAAACGA 638 bp

CTTGATCTCCAGCCATAATTGGTGG

RESULTS

Table (1): Incidence of subclinical mastitis in the examined quarter milk samples (QMS) using California mastitis test (CMT).

Animal species	No. of QMS	Normal QMS		Subclinical QMS	
		No.	%	No.	%
Cow	230.0	130.0	56.5	100.0	43.5

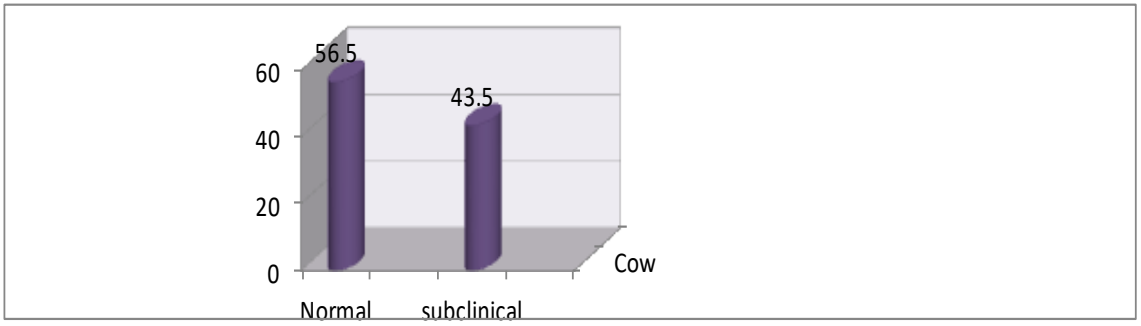

Fig. (1): Incidence of subclinical mastitis in the examined quarter milk samples (QMS) using CMT.

Table (2): CMT scores in the examined quarter milk samples.

Animal Species	Total No. of QMS	Negative samples		POSITIVE samples		CMT scores					
						+		++		+++	
		No.	%	No.	%	No.	%	No.	%	No.	%
Cow	230.0	130.0	56.5	100.0	43.5	12.0	12.00	42.0	42.00	46.0	46.00

Score (+) = weak positive

Score (++) = positive

Score (+++) = strong positive

Table(3): Frequency distribution of subclinical mastitis among the affected quarters according to CMT.

Animal species	Total No. of positive QMS	R.F.		R.H.		L.F.		L.H.	
		No.	%	No.	%	No.	%	No.	%
Cow	100.0	7.0	7.00	29.0	29.00	17.0	17.00	47.0	47.00

***R.F. = Right Fore, *R.H. = Right Hind, *L.F. = Left Fore, * L.H. = Left Hind**

Table (4): Incidence of isolated *S. aureus* depending on the results of Coagulase and TNase tests.

No. of isolates	No. of Coagulase positive isolates	%	No. of TNase positive isolates	%	No. of Coagulase and TNase positive isolates	%	CNS	
							No.	%
169.0	133.0	78.7	135.0	79.9	115.0	68.05	36.0	21.3

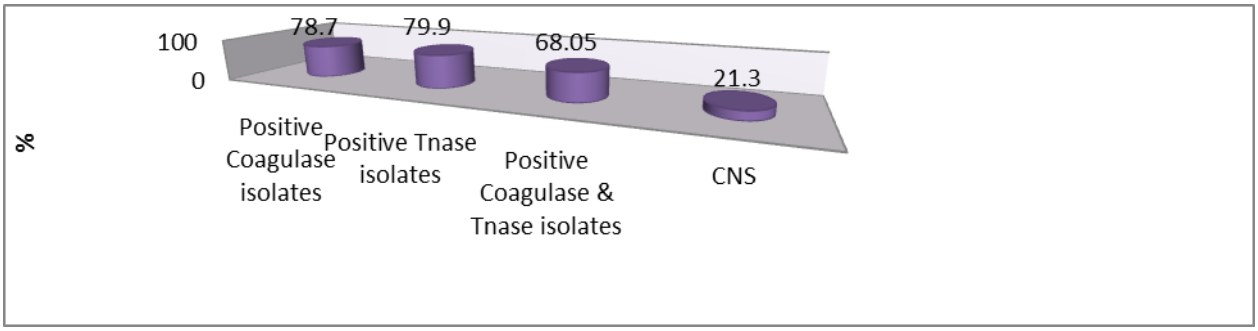

Fig. (2): Incidence of *S. aureus* depending on the results of Coagulase and TNase test.

Table (5): Prevalence of *S. aureus* depending on the results of PCR.

Isolated *S. aureus*		**Examined isolates by PCR**		**Positive PCR isolates**	
No.	**%**	**No.**	**%**	**No.**	**%**
115.0	**68.05**	**5.0**	**4.35**	**5.0**	**4.35**

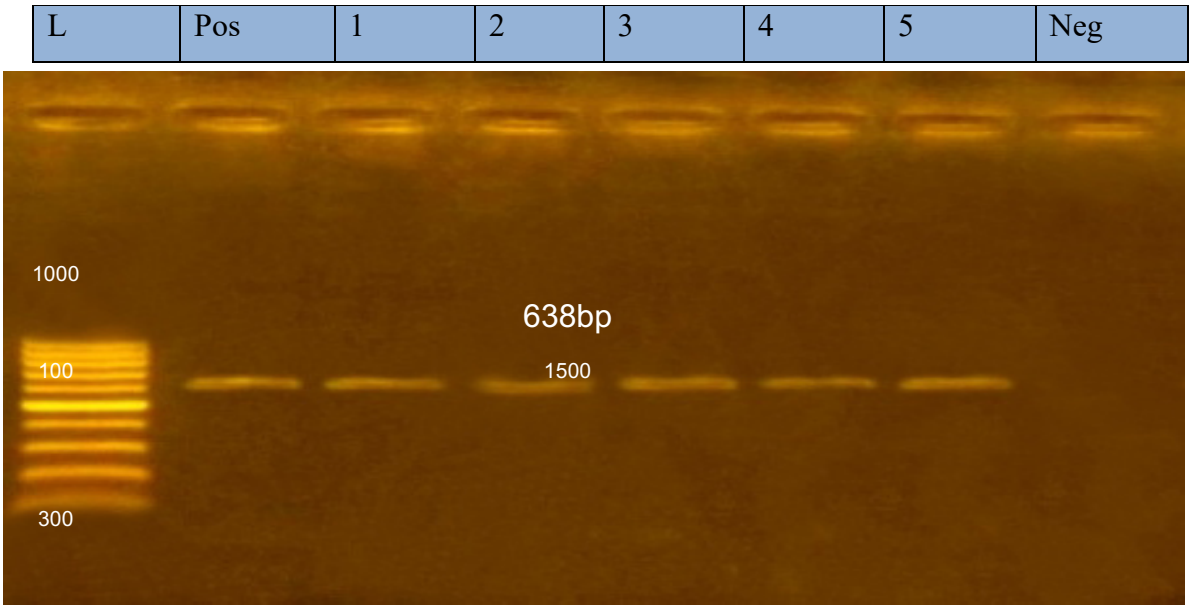

Photo (1): PCR results for *S. aureusc lfA*gene showing positive amplification of 638 bp in the five tested samples.

Lanes 1:5 represent 5 tested samples;Positivecontrol;Neg:Positive control;L

[Gene ruler 100 bp DNA ladder (Fermentas, 100-1000 bp)].

DISCUSSION

1.CMT:

In subclinical mastitis, milk appeared grossly normal and there were no visible signs of inflammation in the udder. However, these are many changes encountered in milk from udder with subclinical infection, cellular, biochemical and enzymatic changes are present in such milk. These changes were detected in most of screening and routine tests applied to diagnose subclinical mastitis cases. The application of screening tests lead to earlier detection of subclinically infected quarter and aid in the selection of dairy animals for either production or therapy **(Zaki *et al*., 2008).** California mastitis test (CMT) is the most widely used test for routine screening of subclinical infected quarters on the farm depending on increased leukocytic count in milk, as a simple, inexpensive and rapid test, the use of CMT to identify infected quarters has been extensively validated **(Khalil, 2007 and Bastan, 2010).**

The data illustrated in Fig. (1) Showed the incidence of subclinical mastitis in the examined quarter milk samples (QMS) using California mastitis test (CMT). The results showed that 130 out of 230 (56.5%) of the examined quarter milk samples reacted negatively to CMT, while 100 (43.5%) of examined quarter milk samples reacted positively. The CMT scores were +ve, ++ve and +++ve displaying the severity of positively reacted samples were 12 (12%), 42 (42%), 46 (46%) of the examined samples, respectively (Table 2). Nearly similar results were reported by **karimuribo *et al*. (2008), abebe *et al.* (2009)**, **amira *et al*. (2013) and Ayano *et al.* (2013). However, Shereen (2009), Neveen (2011), Dabash *et al*. (2014) and Markus *et al*. (2014)** reported higher results**. Meanwhile Zeinhom *et al.* (2013), Kamal *et al.*(2014) and sanitarian *et al*.(2016),** recorded lower results**.** Such variation in CMT scores might be due to the method of milking as manual or automatic, numbers of milking, farm hygiene and some factors relating to milking cows as age, climate, state of udder, number of lactations and labour hygiene. The Frequency distribution of subclinical mastitis among the affected quarters according to CMT, (Table 3) showed that from 100 CMT positive quarters milk samples,7 samples (7%) appeared in right forequarters (RF),29 samples (29%) appeared in right hind ones (RH), 17 samples (17%) in left forequarter (LF) and 47 samples (47%) in left hind (LH). There is higher incidence of subclinical mastitis appeared in the hindquarters than fore ones that is due to unclean area in hindquarters (urination and defecation).

2. Prevalence of staphylococci:

Staphylococcal mastitis are the most common and costly mammary disease of dairy cattle worldwide **(Leitner *et al.,* 2011).** The study was conducted for bacteriological examination of 100 positive CMT quarer milk samples (43.5%) collected from sub clinically mastitic cows. The obtained results showed that all examined samples were contaminated with Staphylococci (Table 4). Nearly similar findings were reported by **Ghaleb *et al.*(2005), Ebrahimi and Akhavan (2009) and Yuan *et al.* (2012). Dutta and Rangnekar (2001), Bedane *et al.* (2012), Marwa and Mahmoud (2013),Hanan *et al.*(2015) and Singh *et al.* (2016),** recorded less findings**.** The presence of Staphylococcus spp. in nearly all samples is probably due to unhygienic milking practices during milking via milker's hands **(Bradley, 2002),** as a result of the dominance of the genus on parts of the human body such as hands, nose, skin and clothing **(Nwagu and Amadi, 2010). El-Attar *et al.* (2002)** considered these organisms as major etiological agents of clinical and sub clinical mastitis worldwide due to teat-to-teat and cow-to-cow spread, possibly via milking machines and perhaps by the miller's hands under the lack of hygiene.

2.1. Prevalence of *S. aureus* in the examined QMS:

Staphylococci could be divided into two groups according to the production of Coagulase enzyme, which is capable of coagulating blood plasma. The synthesis of this enzyme is restricted to some species in the genus, among which *S. aureus*. The other *Staphylococci* that do not synthesize coagulase are referred to as Coagulase negative Staphylococci (CNS) **(Kloos and Bannerman,1995 and Koneman,1997)**. *Staphylococcus aureus* is a major etiological pathogen of bovine mastitis, which triggers significant economic losses in dairy herds worldwide, as it is characterized by persistent and contagious nature. *S. aureus* produces a variety of virulence factors that are responsible for subclinical and persistent intra-mammary infections; some strains of *staphylococcus aureus* demonstrate antibiotic resistance and may persist for longer period without overt symptoms **(Fitzgerald *et al.,* 2000, Aires *et al.,* 2007 and Kahila *et al.,* 2015)**. Regarding the data presented in (Table 5), Fig. (5) showed that, the incidence of *S. aureus* in the examined quarter milk samples depended upon the results of coagulase test. It represented were 78.7%.On the other hand, results of TNase test were 135 (79.9%). whereas the incidence of *S. aureus* depending on both tests was 115 (68.05%). These results agreed with those obtained by **Gianneechini *et al.* (2002), Nam *et al. (*2011)**,

Gitau *et al*. (2013) and Ali *et al.* (2015). Lower results were reported by **Jánosi and Baltay (2004), Sori *et al.* (2005)**, **Bitew *et al.* (2010)**, **Akhtar *et al.* (2012), Zeryehun *et al.* (2013), El-Bagory and Zayda (2015), Mpatswenumugabo *et al*.** (**2017) and Anusha *et al. (*2017**)**. **Radostits *et al*. (2007**) cited that *S. aureus* had a wide ecological distribution being present inside the mammary gland and on the skin which was responsible for higher isolation rate of *S. aureus*. Is a major cause of chronic or recurring clinical mastitis in dairy cows Because of its toxin (poison) production, *S. aureus* can cause mastitis problems ranging from non-clinical infections to clinical or gangrenous infections that may kill the cow **(Roger and John, 2011)**. Development of molecular biological techniques such PCR may be useful significantly as it characterized by accuracy and rapidity in comparison to conventional biochemical test **(Odierno *et al.,* 2006 and Nithin *et al.,* 2012).** (Table 6) determined Polymerase Chain Reaction (PCR) by using specific primer as an accurate and cost- effective molecular diagnostic technique in this study to 4.35% of the isolated *S .aureus,* results showed that positive amplification of 638 bp were obtained in all examined isolates**.** Molecular diagnosis applied to mastitis problems on dairy farms is significantly accurate and cost effective methods of identifying mastitic pathogens, surveillance and control of this economically important disease among dairy cows **(Reyher and Dohoo 2011and Zhao *et al.,* 2006).**

Regarding to the public health hazard, *S. aureus* food poisoning is one of the most common types of food borne diseases worldwide, which caused by an intoxication resulting from the ingestion of food containing Staphylococcal enterotoxins, which is emetic, pyrogenic and mitogenic, suppresses immunoglobulin secretion and enhances toxic shock. *S. aureus* is a common cause of boils, abscesses also more serious affections as endocarditis, osteomyelitis, enterocolitis, toxic shock and scalded skin syndrome**(Stewart *et al.,*2002 and Zadoks, 2003)**.

2.2.Prevalence of CNS in the examined QMS:

Staphylococci are classified into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS) based on the ability to coagulate rabbit plasma.

Although *Staphylococcus aureus* has been described as one of the most important mastitis pathogens in cattle, coagulase negative staphylococci (CNS) are increasingly becoming recognized as etiologic agents associated with inframammary infections (IMI) in most countries. Some consider them as true mastitis pathogens with important virulence factors, a high level of antimicrobial resistance, and the ability to cause chronic infections. Others

regard them as minor pathogens in dairy cows **(Zhang *et al.,* 2000, Gillespie *et al*., 2009, Rajala-Schultz *et al.,* 2009, Taponen and Pyorala, 2009, Supre *et al.,* 2011 and Unal *et al*., 2012).** Table (2) and Fig. (2) Illustrated that coagulase negative staphylococci (CNS) were isolated with the percentages of 21.3% from the examined quarter milk samples. **Anusha *et al.* (2017)** recorded nearly similar results. **Tenhagen *et al*. (2009), and Mpatswenumugabo *et al.* 2017** reported higher findings. **Meanwhile Sampimon *et al.* (2009)** obtained lower results**.** Coagulase negative staphylococci colonize bovine teat skin and teat canals, thus they are classified as skin flora opportunists **(Watts, 1990).** The importance of such bacteria species as a cause of bovine mastitis has come under increased scrutiny in dairy cattle, they were previously considered as mastitis minor pathogens associated with a mild inflammatory reaction but they are now known to cause bovine mastitis **(Bes *et al.,* 2000).**

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