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PCR FOR DETECTION OF VIRULANCE AND ANTIBIOTIC RESISTANCE GENES OF STAPHYLOCOCCUS AUREUS ISOLATED FROM SUBCLINICAL MASTITIS AT AL-GHARBIA GOVERNORATE

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ABSTRACT

Three hundred and fifty quarter milk samples were collected from apparently healthy cows at Al-Gharbia governorate, Egypt. Samples were tested using California Mastitis Test (CMT) and somatic cell count, of which 235 samples were detected as positive. Overall, 168-quarter milk samples (71.48%) were found to be contaminated with coagulase positive *Staphylococcus aureus* with mean count of $64x10^3 \pm 19x10^3$cfu/ml. Isolated *Staphylococcus aureus* strains were tested for methicillin to identify Methicillin resistant strains (MRSA). Antibiotic sensitivity test was carried out by using two antibiotic disks against fourty six identified *Staphylococcus aureus* isolates. The obtained results indicated that resistance against cefoxitin was 34.78% and sensitivity was 65.21%, while the resistance against vancomycin was 26.08% and sensitivity was 73.91%. PCR technique was used to detect presence of mecA gene that coded for penicillin-binding protein 2a.The results on sixteen positive isolates, which suspected to have mecA gene by antibiotic sensitivity test were 93.75%. The total of subclinical mastitis cases infected with MRSA was 6.38%. The results provided evidence that the presence of coagulase positive *Staphylococcus aureus*, as well as Methicillin-resistant strains have become remarkably widespread in subclinical mastitis quarter milk samples. This calls for better control of the sources of milk contamination as well as spread of antimicrobial resistance organisms.

Key words:

Subclinical mastitis, California mastitis test, *Staphylococcus aures* and MRSA.

INTRODUCTION

Raw milk is an excellent medium for growth of several types of microorganisms. Milk and its products are considered vehicles of *Staphylococcus aureus* for infection of humans. It is an important food borne pathogens causing a wide variety of diseases in humans and animals

ranging in severity from mild skin infections to are more severing diseases such as pneumonia and food borne illness. *Staphylococcus aureus* intoxication ranked third of food poisoning cases all over the world **(Asao *et al.,* 2003),** as it is mediated by the ingestion of enterotoxins produced by enterotoxigenic strains of *Staphylococcus aureus* **(Strommenger *et al.,* 2018).** *Staphylococcus aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. In dairy cattle, *Staphylococcus aureus* is frequently associated with subclinical mastitis **(Fagundes *et al.,* 2010).** Cows with subclinical *Staphylococcus aureus* infections can shed a large number of the organism in their milk that can pose an elevated health hazard, resulting in reduction of milk yield by 10 to 20% with undesirable effect on its constituents and nutritional value rendering it of low quality and unfit for processing. Although there are no visible or palpable external changes, the infection is present and inflammation occurred in the udder **(Zdunczyk *et al.,* 2003 and Abdel - Rady and Sayed 2009).** *Staphylococcus aureus* produce a large number of potential virulence factors, which have an important role in the pathogenesis of mastitis **(Kalorey *et al.,*2007).** These include, Coagulase, which is considered the most important virulence factors that clot plasma and coats the bacterial cell, so prevent the phagocytosis **(Panizzi *et al.,* 2004).** Currently, the main therapy for treatment of subclinical mastitis is the administration of antibiotics; but, this approach is associated with a risk of the development of antimicrobial resistant bacteria. *Staphylococcus aureus* has been reported frequently to show multiple antimicrobial resistant patterns. It is detected adverse increasing trend worldwide prevalence of methicillin- resistant strains of staphylococci (MRSA). The infections from methicillin-resistant staphylococci have turned into one of the major problem in antibiotic treatment. The mecA gene, encoding the penicillin binding protein 2a, mediates methicillin- resistance in staphylococci (PBP2a), which has reduced affinity for β-lactamase. The MRSA with mentioned gene is resistant to many other types of antibiotics, this makes the treatment of microorganisms related diseases too hard and causes a greater spread of it in the society **(Pereira *et al.,* 2009).** As MRSA may be present in raw milk and traditional dairy products, this insufficiently hygienic handling of these contaminated foods may lead to transmission of MRSA to human and possible colonization of nostrils, skin and gastrointestinal tract **(Mirzaei *et al.,* 2011).** Many recent MRSA clones have been shown to transmit between animals and humans. Furthermore, MRSA is now invading the hospitals. The MRSA- infected individuals transmit these strains in the hospital setting and result in nosocomial infections **(Kennedy**

and Deleo 2009). Therefore, the aim of the present study was to illustrate the prevalence of subclinical mastitis at El-Gharbia Governorate as well as determination the prevalence of MRSA in the collected subclinical mastitic raw milk samples.

MATERIAL AND METHODS

1- Collection of milk samples according to Andrews *et al.,* 1992:

Three hundred and fifty quarter milk samples were collected from apparently healthy lactating cows in Al-Gharbia Governorate.

2- California Mastitis Test (C.M.T.) According to A.P.H.A., 1992.

Equal volumes of quarter milk sample and CMT reagent **(Schalm *et al.,* 1971)** were mixed thoroughly in a cup of plastic paddle. The mixture was gently swirled by circular motion of the paddle. The results were recorded after 10 seconds and judged. The results were classified into four scores: 0= negative, 1 = slightly positive (+),2 = positive (++) and 3= highly positive (+++).

3-Making Laboratory measurement of milk somatic cells According to Zecconi *et al.,* 2002.

Each positive CMT quarter milk sample was collected under aseptic conditions in a sterile screw caped bottle and sent directly to the laboratory with a minimum of delay to measure the somatic cell count.

4- Phenotypic characterization: Through culturing positive CMT samples and also showing high somatic cell count onto Baired parker agar medium for demonstrating characteristic shape of staphylococci according to **ISO (2003)**, as well as its biochemical and virulence activities was conducted according to **A.P.H.A, 2004.**

5- Detection of mecA gene of *Staphylococcus aureus* by in vitro antibiotic sensitivity test:

Sensitivity to antibiotics was determined by agar diffusion test on Muller Hinton agar using the following antibiotic impregnated discs:cefoxitine (30 µg) and vancomycin (30 µg), Oxoid Germany. Zones of growth inhibition were evaluated according to Clinical Laboratory Standard Institute **(CLS I, 2014).**

6- PCR detection of mecA gene of *Staphylococcus aureus.*

DNA Molecular weight marker

The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded**.**

Agarose gel electrophoreses (Sambrook *et al*., 1989)

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5µg/ml ethedium bromide was added and mixed thoroughly.

RESULTS

Table (1): The prevalence of subclinical mastitis in quarter milk samples collected from lactating cows according to the results of California mastitis test (CMT) (N=350).

Total quarter milk samples	Negative by CMT		Subclinical mastitis by CMT							
			Total positive		Score +		Score ++		Score +++	
	No.	%	No.	%	No.	%	No.	%	No.	%
350	115	32.86	235	67.14	28	11.92	78	33.19	129	54.89

Table (2): Statistical analytical results of milk somatic cell count (SCC) / ml in examined 235 quarter milk samples.

No. of examined samples	Minimum	Maximum	Mean	± S.E.M.
235	$10x10^4$	$3.0x10^6$	$46.0x10^4$	$3.7x10^4$

Table (3): Frequency distribution of the examined 235-quarter milk samples according to their milk somatic cell count (SCC) / ml.

Interval	NO. of samples	%
$<200x10^3$	89	37.87
$\geq 200\ x10^3 - <400\ x10^3$	66	28.09
$\geq 400\ x10^3 - <600\ x10^3$	21	8.94
$\geq 600\ x10^3 - <800\ x10^3$	20	8.51
$\geq 800\ x10^3 - <1000\ x10^3$	16	6.81
$\geq 1000\ x10^3$	23	9.78
Total	235	100.00

Table (4): Prevalence and statistical analysis of *Staphylococcus aureus* and MRSA in the examined quarter milk samples.

No. of examined samples	Coagulase positive Staphylococci samples		Mean (Cfu/ml)	±SEM (Cfu/ml)	Positive Staphylococcus aureus samples		Positive MRSA Samples	
	NO.	%			NO.	%	No.	%
235	168	71.48	$64x10^3$	$19x10^3$	45	19.14	14	31.11

Table (5): Results of Mannitol Salt Agar test for differentiation between Coagulase positive Staphylococci isolates.

Tested coagulase positive staphylococcus isolates	*S. aureus*		*S. intermedius*		*S. hyicus*	
No.	No.	%	No.	%	No.	%
383	46	12.01	222	57.96	115	30.03

Table (6): Detection of mecA gene by Antibiotic Senstivity Test.

Tested *Staphylococus aureus* isolates	Cefoxitine				Vancomycin			
No.	Resistant		Sensitive		Resistant		Sensitive	
	No.	%	No.	%	No.	%	No.	%
46	16	34.78	30	65.22	12	26.09	34	73.91

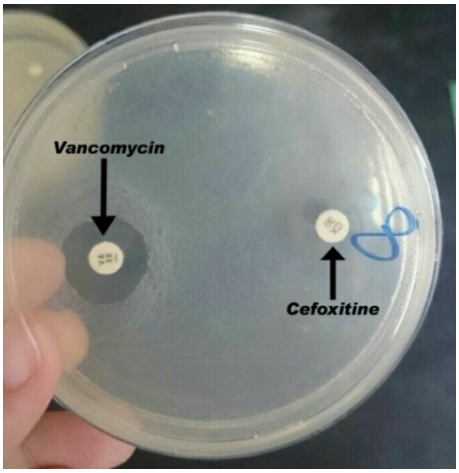

Fig. (1): Antibiotic sensitivity test showing resistance to cefoxitine and sensitivity to vancomycin which suspect to have mecA gene of *Staphylococcus aureus.*

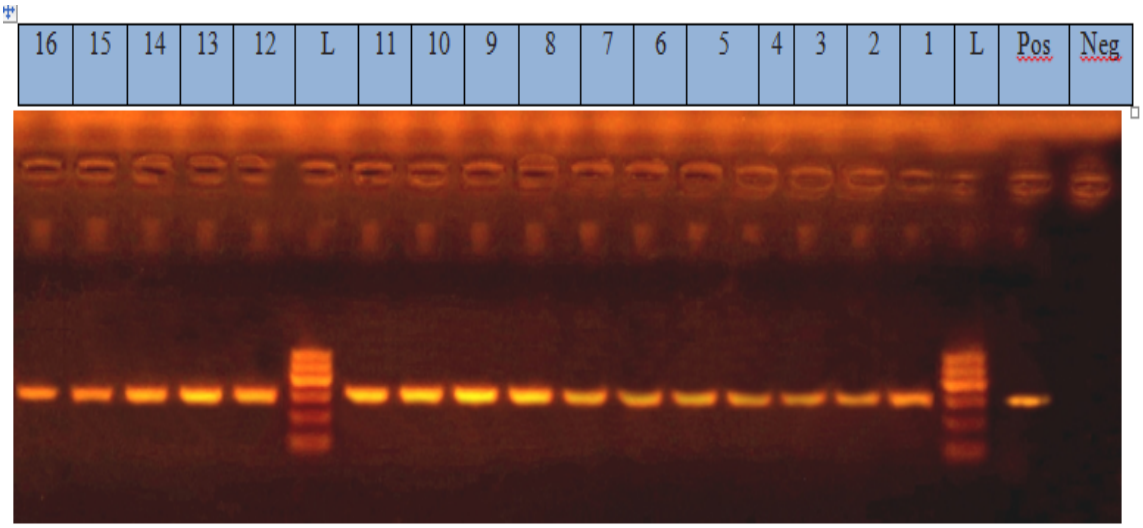

Fig. (2): Results of PCR indicate the presence of mecA gene in the identified Staphylococcus aureus isolates.

DISCUSSION

Mastitis in dairy cows is a serious problem as it is an economically devastating disease causing great economic losses in the dairy industry in Egypt **(Seleim *et al.,* 2002)**. Subclinical mastitis is more serious than clinical one because visible abnormalities such as udder swelling, hardness of the affected quarter, pain, and watery milk remain absent. It results in reductions of milk yield and undesirable changes in the milk composition, as well as increased costs associated with control strategies **(Halasa *et al.,* 2007).** Data represented in (Table 1) revealed that, the results of examined 350 quarter milk samples by California Mastitis Test was 115 (32.86%) samples were negative, while 235 (67.14%) samples were positive. Among the positive samples the highest incidence was recorded in CMT (+++) as129 (54.89%) samples; while the lowest one lied in CMT (+) as 28 (11.91%) samples. These results totally agree with those reported by **Mulei (2000) and Kerro and Tareke (2003)** while lower incidences were obtained by **Elbably *et al.* (2013); Lucia *et al.,* (2017) and Xavier *et al.,* (2017). Kalorey *et al.* (2007)** recorded higher incidence of subclinical mastitis using CMT (90.76%).The difference in prevalence of subclinical mastitis observed may be due to differences in management practices, use of different methods for diagnosing subclinical mastitis, breeds of the animals, immune responses and climatic conditions. Other factors that could influence the prevalence of subclinical mastitis could be attributed to variation in hygienic standards of the dairy environment and milking conditions, as well as genetic variation in disease resistance amongst the breeds maintained in the systems **(Saidi *et al.*2013).** (Table 2) showed that somatic cell count of the examined quarter milk samples was in the range of $10x10^4$ to 3.0x106 with a mean value of 46.0x104 ± $3.7x10^4$. Increase in somatic cell count in milk leads to the release of lipolysis (lipases) and proteolysis (plasmin) enzymes, which can degrade the triglycerides of milk fat and casein contents of milk. Leading to poor quality milk in the mastitis-affected animals **(Ondiek *et al.* 2013).** The highest frequency distribution of milk somatic cell count was 65.96% (155 samples), lied within the range $<200x10^3$- $<400x10^3$cells/ml (Table 3); which substantiate what have been reported by **Nam *et al.,* (2010)and Dieser *et al.,* (2014)**. Statistical analytical result of Coagulase Positive Staphylococci was present in a percentage of 71.48 % of the examined raw milk samples with a mean value of $64x10^3$ ± $19x10^3$ (Table 4). **Lucia *et al.,* (2017),** recorded lower results. The production of the coagulase enzyme becomes the pathogenic factor of *Staphylococcus*

aureus, differentiating it from other types of Staphylococci, which is able to clot blood plasma, since it resembles prothrombin, which can convert fibrinogen to fibrin **(Lucia *et al.,* 2017).** In addition, results in (Table 4) showed that, the incidence of *Staphylococcus aureus* was 19.14% from the examined raw milk samples. **Sharma *et al.,* (2015),** obtained nearly similar results; **Fagundes *et al.,* (2010) and Umaru *et al.* (2017)** recorded lower results**,** while higher incidences were recorded by **Abdel-Rady and Sayed (2009)**. According to the results reported in table 4 , out of 45 coagulase positive *Staphylococcus aureus* samples detected, 14 (31.11%) were found to contain mecA gene which is indication of presence of methicillin resistant *Staphylococcus aureus* (MRSA) by using PCR technique, **Guimarães *et al.* (2017)** recorded higher results. Data represented in (Table 5) shows that, out of 383 tested isolates of Staphylococci, 46 were *Staphylococcus aureus* in apercentageof 12.01%, whileCoagulase Positive *Staphylococcus intermedius* was found in percentage of 57.96%. Coagulase Positive *Staphylococcus hyicus* was found in a percentage of 30.03 %. The prevalence of *Staphylococcus aureus* can most likely be attributed to the wide distribution of the organism inside the mammary glands, as well as on the skin of teats and udders **(Anueyiagu *et al.,* 2016)**. The data obtained in (Table 6) showing in vitro antibiotic sensitivity test of the examined isolates to different antibiotics, which revealed that 16 isolate (34.78%), were resistant to cefoxitine. Similar results obtained by **Sharma *et al.,* (2015)**, while lower results were obtained by **Hamid *et al.,* (2017). Dorgham *et al.,* (2013)** recorded very high results of resistance to cefoxitine. The resistances of isolates to vancomycin were present in a percentage of 26.09 %while 34 samples (73.91%) were sensitive. **Al-Ashmawy and Sallam (2016)** obtained similar results. Fourteen isolates were resistant to cefoxitine and sensitive to vancomycin, which were suspected to have mecA gene, while two isolates only were resistant to cefoxitin, and vancomycin. In other studies carried out on cow's milk, MRSA were most frequently isolated from milk of animals showing signs of subclinical mastitis **(Lee 2003).** Antimicrobial resistance represents a serious problem in the treatment of infectious diseases including mastitis. In recent years, an increasing antimicrobial resistance rate has been recognized in *Staphylococcus aureus* from bovine mastitis **(Saini *et al.,* 2012 and Wang *et al.,* 2013).** Moreover, there is an increased incidence of Methicillin resistant *Staphylococcus aureus* (MRSA) all over the world, which seems to be widely spread among *Staphylococcusa ureus* isolates from bovine milk. MRSA first emerged as a serious pathogen in human medicine during late 1970s and has been reported in animals during the past 10

years **(Leonard and Markey, 2008).** With the emergence of MRSA, methicillin became ineffective against them while vancomycin became the drug of choice for MRSA **(Ng *et al.*, 2011).** By using PCR for detecting the mecA genes in 16 *Staphylococcus aureus* isolates, the results showed that 15 out of 16 positive isolates by antibiogram method (93.75%) contained mecA gene. **El-seedy *et al.* (2010) and Hamid *et al*. (2017)** obtained lower results. In conclusion, the results obtained in this study confirmed that *Staphylococcus aureus* was implicated in subclinical mastitis in the examined quarter milk samples, of which high incidence of MRSA are present representing major threats for transmission to human beings. Thus to safe guard consumers from being infected, improving the hygienic conditions and careful handling of the producing animal during milking must be applied.

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