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Molecular and Bacteriological Investigation of Contagious Mastitis Caused by *Staphylococcus aureus* in Dairy Cattle Farms in Egypt

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Abstract: Contagious mastitis in cattle, caused by *Staphylococcus aureus* (*S.aureus*) is considered of very indispensable significance to the public health everywhere throughout the world as well as in Egypt. Mammary glands with subclinical mastitis can shed vast quantities of *S. aureus* in milk. Emerging of multidrug resistant strains, the capacity to form biofilm in vivo and zoonotic part of *S. aureus* is considered of very fundamental significance to the public health around the world. The point of this examination was to explore the predominance of subclinical contagious mastitis caused by *S. aureus* on small-scale dairy farms in Giza and Al-Kalubia governorates of Egypt. A sum of 360 quarter milk samples were gathered from 90 apparently healthy dairy cows. Of them, 172 quarter milk samples (47.77%) were positive for subclinical mastitis (SCC > 200 x10^3cells/ml) utilizing Somatic Cell Count (SCC), as a pointer for subclinical mastitis (SCM). *S. aureus* was distinguished by phenotypic strategies from 55.81% (96/172) of the positive subclinical mastitis milk samples. Further genotypic techniques for 23S rRNA uncovered that out of the 96 bacteriologically recognized isolates 93 were affirmed as *S. aureus*. In conclusion, *S. aureus* was affirmed as the most dominating among contagious mastitis causing pathogens in Egypt. The high occurrence of *S. aureus* mastitis in Egypt is primarily because of improper hygienic and poor farm management. A combination of phenotypic and genotypic tests is prescribed for diagnosis of *S. aureus*.

Key words: Contagious Mastitis · *Staphylococcus aureus* · Somatic Cell Count · Subclinical Mastitis · PCR.

INTRODUCTION

In Egypt, more than 70 % of the aggregate domesticated animals are owned by subsistence and little-scale farmers, who keep couple of animals as a source of milk and dairy products for home utilization or to offer, frequently unpasteurized, in nearby markets [1].

Regardless of endeavors applied in the control and counteractive action of mastitis by the execution of udder health programs, mastitis is as yet the most pervasive and costly infection in dairy production from the financial, diagnostic and public-health related perspectives [2, 3]. Bovine mastitis is a financial weight for agriculturists particularly in its subclinical form due to diminished milk yield and changes in milk composition, premature culling, cost of veterinary medications and renders drain unfit for human utilization [4]. Besides, cows experiencing subclinical mastitis are source of infection for other animals [5, 6].

Around 150 species of microorganisms, for the most part bacteria, can cause mastitis [7]. They are separated into two gathering: contagious and environmental udder pathogens. The fundamental contagious mastitis pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma spp.* Their essential supply is the bovine's udder and they can spread from cow to cow at or around the season of draining [8], in this manner, when they are found in bulk milk, these mastitis causing organisms are solid markers of the nearness of intramammary contaminations in the herd [9].

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Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium, causes clinical and subclinical mastitis in dairy cows and is normally connected with rising somatic cell count (SCC). In addition, therapeutic medication of *S. aureus* mastitis (SAM) during lactation seasons is normally unsuccessful or with poor achievement and sometimes is misleading [10]. This may incite a positive possibility for spread of infection among the producing animals; in this way expanding the financial losses of SAM specially in small-scale cultivating with sublevel of clean measures prompting a moderately high culling rate. It has been discovered that *S. aureus* is in charge of more than 80% of subclinical bovine mastitis, which may bring about $300 loss per animal [11]. Besides, *S. aureus* secretes few enterotoxins and a toxic shock syndrome toxin-1 (TSST-1), henceforth its existence in milk causes severe food poisoning and represents a public harming for purchasers [12].

The early, quick and effective identification of bovine mastitis remains a noteworthy bottleneck and is of most extreme significance that specifically impacts the speed with which treatment choices and administration are embraced for infection control. The early identification of mastitis may build the cure rate by 60 % and decrease the time needed to recover optimum milk yield when joined with fitting antimicrobial treatment [13].

Various investigative strategies for mastitis and recognizable proof of the mastitis causing microbial operators, including California Mastitis Test (CMT), Somatic Cell Count (SCC), bacteriological and molecular analysis are accessible. Bacteriological investigation could recognize the microbial pathogens, however it is costly, tedious and is not profoundly particular [14]. Then again, molecular inspection in view of DNA intensification of the objective pathogen sole DNA sequences within the 16S or 23S subunit of the ribosomal RNA (rRNA) has been ended up being a precise, solid and quick diagnostic strategy [15, 16].

The point of this study was to explore the predominance of contagious mastitis caused by *S. aureus* in randomly chosen cattle, which were sampled from households and small-scale dairy farms in Giza and Al-Kalubia governorates of Egypt. In addition, assessment of the different diagnostics for mastitis particularly in subclinical cases. lastly, genotypic characterization of the contagious mastitis causing pathogens that incite proficient treatment and control of bovine mastitis.

MATERIALS AND METHODS

Ethical Approval: The approval from the Institutional Animal Ethics Committee to carry out this study was not required as no invasive technique was used. The milk samples were collected from apparently healthy lactating cows.

Animals: A total of 90 apparently healthy dairy cows with normal milk secretion, at different stages of lactation from households and small-scale dairy farms during the period 2016-2017, located in different areas in Giza and Al-Kalubia governorates, Egypt. These animals were subjected to clinical examination for detection of any clinical abnormalities with special attention to the udder by visual inspection and palpation for detection of clinical mastitis according to Kelly [17].

Samples: A total of 360 quarter milk samples collected from 90 apparently healthy dairy cows were employed in this study. Quarter milk sample was collected aseptically following the guidelines of the National Mastitis Council [18], as 15 ml of milk were collected from each quarter in sterile tube under strict hygienic measures from each quarter after disinfection of the teat with 70% alcohol. The first 3 squirts from each quarter were discarded. Milk samples were kept on ice and transferred immediately to the laboratory for assessment of SCC and bacteriological examination within 24 hours.

Somatic Cell Count (SCC): Automatic somatic cell counter (NucleoCounter SCC-100) was used to determine SCC of milk samples. Initial warming of samples at 35°C for 5 minutes was accomplished in a water bath before mixing and reading [19].

Bacteriological Examination of Milk Samples [20, 21]: The collected milk samples were either incubated for 18-24 hours at 37°C or milk samples were directly incubated for 24 hours at 37°C in peptone water, then all samples were cultivated on Mannitol Salt Agar (selective media for *Staphylococci*) and Blood Agar (for detection of haemolysis). All plates were incubated at 37°C for 18-24 hours and examined for bacterial growth. The colonies were examined for their morphological characters, appearance and hemolytic activity. Smears from suspected colonies were prepared and stained with Gram's stain to be examined microscopically before being transferred into semisolid slope agar for further identification.

Identification of the Isolates [20]: Isolated staphylococcal colonies were further identified. Briefly, pure culture of isolated organisms was prepared then subjected to microscopical examination and Biochemical identification [22].

Molecular Identification of the Bacterial Isolates Using Polymerase Chain Reaction (PCR): For molecular identification and confirmation, the isolated bacteria were further cultured in TSB (Difco Laboratories, Detroit, Mich.) at 37°C for about 17 h before DNA extraction. Bacterial DNA was extracted by GF-1 Tissue DNA extraction kit (Vivantis Co., Malaysia). The oligonucleotide primers for *S. aureus* species specific regions of the DNA coding for 23S rRNA for *S. aureus*) using Sau327 5′-GGACGACATTAGACGAATCA-3′ and Sau1645 5′-CGGGCACCTATTTTCTATCT-3′ [14].

All reactions were carried out in a final volume of 25 µl. Volumes of 200 ng of extracted DNA template or 2 µl of bacterial DNA, 0.6 µl (100µ mol) primer, 5 µl of Taq PCR Master Mix (5x FIREPol® Master Mix Ready to Load, Cat. no.04-12-00115, Solis Biodyne Co.) were used in the reaction.

A pre-PCR step at 95°C for 2 min was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 45s, annealing at 62°C for 1min and extension at 72°C for 2min. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. The PCR products were analyzed and visualized through electrophoretic separation on 1.5% agarose gel.

The positive PCR products were then purified and sequenced in both directions on an ABI Prism 3100 Genetic Analyzer using a Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems). Identification was achieved by comparison of edited sequences against GenBank with Blast [23].

Nucleotide Sequence Accession Numbers: The nucleotide sequence data reported in this paper was submitted in the GenBank nucleotide sequence databases with the accession numbers MG372745, MG372746 and MG372747 for 23rRNA gene.

RESULTS

Three hundred and sixty quarter milk samples gathered from 90 apparently healthy dairy cows with normal milk secretion were examined for subclinical mastitis through evaluation of SCC. Of these 360 quarter milk samples, 172 milk samples (47.77%) were positive for subclinical mastitis (SCC > 200 x10^3cells/ml). The assessed SCC in the subclinical mastitis samples was 500x10^3-1500 x10^3cells/ml in 110 milk samples (63.9%), 200x10^3-500x10^3 cells/ml in 56 milk samples (32.6%) and 1500 x10^3-5000 x10^3 cells/ml in 6 milk samples (3.5%) (Table 1).

Conventional bacteriological and biochemical examination of the positive SCM milk samples uncovered the separation and distinguishing proof of *S. aureus* from 96 milk samples (55.81%) (Table 2).

Molecular identification using PCR to affirm the detached *S. aureus* isolates confirms 93 *S. aureus* isolates (54%) as appeared in Table (2). The PCR gave the expected PCR product for *S. aureus* (1318bp) as shown in Fig. (1).

Clear sequences of the 23S rRNA were gotten from all the isolates. Homology search proved that all isolates were *S. aureus*.

Table 1: Prevalence of subclinical mastitis using SCC in the examined dairy cow quarter milk samples

Test	SCC (cells/ml)	No of quarter milk samples	SCC range	No & %
SCC	< 200x10^3	188 (52.23%)	10 – 200 x10^3	188 (52.23%)
	> 200x10^3	172 (47.77%)	200-500 x10^3	110 (63.9%)
			500-1500 x10^3	56 (32.6%)
			1500-5000 x10^3	6 (3.5%)
Total		360		360 (100%)

Table 2: Results of *S. aureus* identification by conventional bacteriological and molecular diagnosis in SCM positive quarter milk samples (172 samples)

		S. aureus Diagnosis in SCM samples			
		Bacteriological diagnosis		Molecular diagnosis	
Total No of milk samples	SCM (SCC > 200x10^3)	Positive	Negative	Positive	Negative
360	172 (47.77%)	96 (55.81%)	76 (44.19%)	93 (54%)	79 (46%)

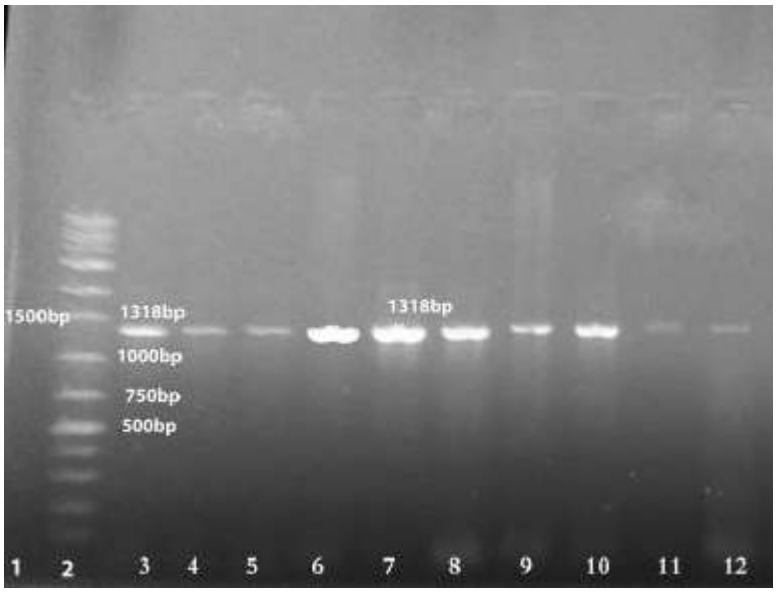

Fig. 1: PCR amplified products among the examined *S. aureus* isolates.
Lane (1), negative control; Lane (2), 100 bp marker; Lane (3), positive control for *S. aureus*; Lanes 4-12, positive for *S. aureus* isolates.

DISCUSSION

Mastitis is a standout amongst the most vital dangerous infectious diseases of dairy cows industry [24] came about because of ascending infection by numerous mastitis pathogens. However, two principle classes were prevailed; contagious and environmental pathogens. *S. aureus* and *Str. agalactiae* are viewed as the principle causative agents of contagious mastitis, while coliform organisms and other streptococci are those related with environmental mastitis [25, 26].

The public health risk of contagious mastitis is ascribed to be related with numerous zoonotic diseases in which milk goes about as a vehicle for the infectious agents [27].

As of late, awesome consideration has been paid toward enhancing the analysis of mastitis especially in the early subclinical frame. Of those strategies utilized for subclinical mastitis are somatic cell count estimation either directly by automatic somatic cell counters, or indirectly by implication over recording gel formation comes about because of the collaboration between nuclear material of somatic cells excreted in milk and particular compound reagent (key of California Mastitis Test) [28].

Since somatic cell count (SCC) in milk has been considered as an unrivaled marker for subclinical mastitis, the utilization of SCC is basic and vital for deciding the diminishment of the milk production [29, 30].

Sound bovine's milk contains low levels of SCC and regularly runs from 50,000 to 100,000 cells/ml and the SCC edge for human utilization is 200,000 cells/ml and milk contains SCC increasingly that this esteem is viewed as unhealthy for human utilize [31]. In a similar vein, European nations have confined the human utilization of milk with SCC to not surpass 400,000 cells/ml.

In the present work, 360 quarter milk samples gathered from 90 clearly healthy dairy bovines with ordinary milk secretion were analyzed for somatic cell content, as a pointer for subclinical mastitis. 172 quarter milk samples (47.77%) were certain for SCM (SCC > $200x10^3$/ml) as appeared in Table (1). These discoveries are about like that recorded by Mdegela *et al.* [32] (51.6%), Sayed *et al*. [33] (56.3%) and somewhat not as much as that of EL-Rashidy *et al.* [34] (62.08%), Awad [35] (69.4%).Meanwhile plainly higher prevalence was recorded by Elhaig and Selim [36] (71.6 %), Karimuribo *et al.* [37] (75.9%), Kivaria *et al.* [38] (78%) and Elsayed *et al.* [39] (91.48%) between the dairy cow.

Despite what might be expected, these discoveries can't help contradicting that revealed by Abou-Zaid and Bahout [40] (20.6 %) and Lakew *et al.* [41] (32.7%) of dairy bovines have subclinical mastitis.

In the present work the SCM positive samples were inspected for *S. aureus* prevalence, this was parallel to Sayed *et al.* [33].

S. aureus has been considered as one of the major bacterial causative agents of contagious mastitis [42] and furthermore for the highest seriousness of the mastitis warmth [43]. Thus, declining of this serious pathogen is of awesome results.

As the present gold standard is as yet the bacterial culture as revealed by Elhaig & Selim [36], consequently the existing investigation expected to estimate the predominance of Contagious Mastitis utilizing bacteriological and molecular strategies as a productive and supplementary instrument for diagnosis of contagious mastitis causative agent in dairy herds.

Bacteriological examination of the SCM positive milk samples (Table 2) uncovered the segregation and distinguishing proof of *S. aureus* isolates from 96 (55.81%) quarter milk samples (Table 2). Another study affirms the isolation of *S. aureus* from 80% of subclinically mastitic dairy cattle [44]. Our results of the high extent of *S. aureus* in SCM cases harmonize with that of past investigations [32, 45]. Unexpectedly, these discoveries can't help contradicting that detailed by Sayed *et al*. [33] (14.8%) of dairy bovines experienced subclinical mastitis.

However microbial segregation of the causative microorganism(s) is the most exact technique for mastitis discovery, it is costly and tedious. In this way, the requirement for a basic very touchy, quick and dependable test adequate to be applied on vast size of animals is in this way required [24].

The PCR, as an alternative diagnostic test to bacterial separation, has preferences over bacterial culture get from its capacity to distinguish the low number of pathogens, even in a dead state, simple handle samples at the same time and in addition, its velocity, affectability, simplicity of investigation and elucidation[36]. Moreover, PCR can be utilized routinely for the early location of animal reservoirs and gives the veterinarian and the owners the outcomes rapidly with expanded affectability, therefore bring down the cost of treatment and enhance mastitis administration [46, 47].

Molecular examination utilizing PCR for the bacteriologically secluded and recognized *S. aureus* segregates demonstrated 93 *S. aureus* isolates (54%) as appeared in Table (2). The PCR gave the expected PCR product for *S. aureus* (1318bp) as appeared in figure (1).

From Table (2), the PCR utilized, neglected to distinguish three bacteriologically recognized *S. aureus* isolates. This might be clarified that the sensitivity of PCR test in the distinguishing of bacterial pathogens relies upon the primers utilized and the identification of nucleic acids rather than live cells other than the associated hindrance with the PCR reaction [47]. In any case, there are still high understanding between results of bacterial culture and PCR.

Because of the constraints of microbial cultivation techniques, the improvement of polymerase chain reaction (PCR)-based strategies can be utilized as an option fast and touchy diagnostic instrument to recognize contagious mastitis pathogens, giving a promising choice to the quick identification made in hours, as opposed to days devoured by ordinary bacteriological techniques [48].

CONCLUSIONS

All in all, this investigation demonstrates that *S. aureus* was affirmed as the most transcendent among contagious mastitis causing pathogens in Egypt utilizing bacteriological and molecular systems. The high occurrence is usually because of improper hygienic and poor farm management of small-scale farms.

A mix of phenotypic and genotypic tests is prescribed for researching *S. aureus*. Molecular technique indicated high specificity (96.88 %) for analysis of *S. aureus* with focal point of time saving.

Encourage epidemiologic examinations are expected to research the hereditary assorted variety of *S. aureus* utilizing a large number of isolates gathered from various territories.

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