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Assessment of Conventional and Molecular Features of *Staphylococcus aureus* Isolated from Bovine Milk Samples and Contact Dairy Workers

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Abstract: A total of 384 apparently normal quarter foremilk samples, collected from 96 machine milking lactating Friesian cows from Aswan governorate, were cultivated on Baird Parker media. 52 swabs collected from contact dairy workers' hands or faces were streaked on mannitol salt egg yolk agar (MSEY agar). Isolated *Staphylococcus aureus* strains were examined for coagulase slide test, Catalase and DNAase test. *Staphylococcus aureus* isolates were tested by a two multiplex PCR assays one of them was to detect 16SrRNA gene and coagulase (coa) gene. The second multiplex PCR was to clarify presence of *S. aureus* entertoxins type A (SEA) and B (SEB) in positive coagulase *S. aureus*. For bovine milk, a prevalence rate of 39.7 % and 34.1 of California mastitis test (CMT) and Somatic cell count (SSC) was detected, respectively. Whereas, coagulase test was positive in 33.55 and 56.52% of bovine milk and contact dairy workers in the same sequence, 16Sr-RNA gene was positive in 49.61 and 82.6% of bovine milk and dairy workers, respectively. While coagulase (coa) gene was positive in 40.45 and 69.56 % of milk and dairy workers, respectively. SEA was positive in 21.37 and 43.47 % of milk and dairy workers in the same manner, respectively. While SEB was positive in 20.61 and 34.78% in the same sequence, respectively. In conclusion testing milk (by CMT, SCC and microbial isolation) for detection of subclinical mastitis is an essential part of daily evaluation record of each dairy farm and detection of *S. aureus* by PCR as well as entertoxins identification found to be rapid, sensitive and accurate. Special concern should be directed to elucidate the kinetic transfer of *S. aureus* between animals and human. Detection of *S.aureus* entertoxin A and B were found to easy and rapid by using PCR.

Key words: *Staphylococcus aureus* · Bovine milk · Dairy workers

INTRODUCTION

Staphylococcus aureus is the world over pathogen that causes a variety of infections in man and animals as bovine mastitis which may be clinical or subclinical. Subclinical mastitis occurred when no visible or palpable external changes can be defined, despite the occurrence of the udder infection [1]. *S. aureus*, a contagious pathogen that can be easily Transferred from cow to cow, usually manifests as subclinical mastitis with an elevated somatic cell count (SCC) in milk and is associated with decreased milk quality [2].

Prevention of bovine mastitis and production of high quality milk are strategic to favorable development of dairy business and proper response to consumer demand [3].

S. aureus is responsible for one of the most common types of food poisoning due to the ingestion of food or beverage containing one or more performed enteroxins (SE) produced by *S. aureus* [4].

Five classical staphylococcal enterotoxins (SE) SEA–SEE have been recognized and sporadic cases as well as outbreaks due to these enterotoxins are described [5, 6]. Recently, new SE was identified: SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ and SEU [7-9].

The persistence of *S. aureus* and the poor response of pathogen to antibiotic therapy make *S. aureus* a common cause of animal culling. Many attempts have

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been made to develop effective vaccines against *S. aureus*, but most vaccines do not reduce the incidence of infection [10].

On dairy farms producing high quality milk whereas, other types of mastitis are controlled, *S. aureus* continues to be an important and seemingly ubiquitous mastitis pathogen [11]. *S. aureus* can gain access to milk either by direct excretion from udders with clinical and subclinical staphylococcal mastitis or by contamination from housing materials, fodder, equipment, air, bovine skin, non-bovine animals and humans [12].

Food safety standards include the surveillance of *S. aureus* and, according to food type, different contamination levels have been established. Routine detection of *S. aureus* in food is usually carried out by traditional methods based on the use of selective media, like Baird-Parker for direct enumeration or the recovery of isolates after enrichment in selective broth for 24-48hours at 37°C. Following, suspicious colonies should be identified by biochemical test as DNase and coagulase production. However, this conventional methodology takes many days [13].

PCR-based techniques are being increasingly used for identification and typing since they are rapid and easy to test large numbers of strains with a high reliability and differentiation power. A number of these techniques have been applied to the genus Staphylococcus for species identification like PCR-based DNA fingerprinting,16S-23S r DNA as well as typing of *S. aureus* isolates by randomly amplified DNA has been applied to the genus Staphylococcus species for typing of *S. aureus* isolates [14].

PCR is used for the detection of food-borne pathogens to replace the time-consuming culture-based classical techniques [8]. These techniques are rapid, easy to handle, sensitive and specific and therefore constitute very valuable tools for routine applications. Specific primers for PCR detection of *S. aureus* have been directed to enterotoxin genes [15]. Detection of the staphylococcal enterotoxin genes have been also approached by PCR in multiplex reactions [13,15]. They were proved as rapid, specific, sensitive and reliable methods when compared to immunological toxin production assays.

The objective of this study was to detect *S. aureus* in collected milk samples from apparently normal animals, comparing genotypic traits of *S. aureus* strains isolated from cattle bulk-tank milk samples and those isolated from hands of contact dairy workers. So, testing of isolated *S. aureus* by two multiplex PCR assays for 16 SrRNA gene and coagulase gene. All positive coagulase *S. aureus* were tested for classical staphylococcal enterotoxins SEA and SEB.

MATERIALS AND METHODS

Milk Samples: A total of 384 apparently normal quarter foremilk samples were collected from 96 machine milking lactating Friesian cows of different ages and numbers & stages of lactations from Aswan governorate, Egypt. Examination of udder and teats was done according to Radostitis *et al.* [16]. The examination of milk was done according to Schalm *et al.* [17] by drawing first streams of milk over a black surface for detecting of watery milk, color change, presence of flakes or clots. Milk samples were transported in an icebox. In the laboratory each sample was divided into 3 sterile vials, the first for California mastitis test (CMT) application according to Schalm *et al.* [17] and APHA [18] it was done at farm, the second for somatic cell count SSC according to Zecconi *et al.* [19], using automatic reader *Bently Soma* count. Last part used for bacteriological examination.

***S. aureus* Isolation:** From 384 milk, swabs samples selectively cultivated on Baird Parker media, its colonies were black in color and surrounded by a clear zone (due to lecithinase activity) [12].

Conventional Biochemical Identification: Identification of grown culture *S. aureus* was carried by gram stain followed by further biochemical characterization including: catalase test, slide coagulase test and the production of thermo-nuclease (on DNase test agar, Oxoid) [13].

Contact Dairy Workers: Swabs from 52 contact dairy workers were collected either from any wounds or abrasions on worker hands or face. Collected swabs were subjected to direct and enrichment culture methods. For direct culture, hand swabs were streaked on mannitol salt egg yolk agar (MSEY agar). For the enrichment culture, hand swabs were cultured in Tryptic soy broth (Nissui) containing 7.5% NaCl at 35°C for 22-24 hours. Then loop-full of culture was streaked on MSEY agar. After incubation of MSYN at 35°C for 24-48 hours, bright yellow colonies were picked and subjected to further analysis as those used for isolates of *S. aureus* from milk [20].

Molecular Assessment of Isolated *S. aureus*: All the isolates were cultured for DNA preparation by incubation overnight in 10 ml brain heart infusion broth (Oxoid), centrifuged (5000 g., 15 min.) and re-suspended in 0.5 ml TE (10 mM Tris, 1 mM EDTA) [pH 8].

Table 1: Oligonucleotide primer and the corresponding reaction conditions

Primer	Sequence	Reaction conditions	Product size	References
16srRNA F	GTA GGT GGC AAG CGT TAT CC	Initial devaluation 93°C for 3 minutes	228	[24]
16srRNA R	CGC ACA TCA GCG TCA G	followed by denaturation at 92°C for		
		1 minute Anneal. at 52°C for 1 minute		
Coa F	5'-ATA GAG ATG CTG GTA CAG G-3'	extension at 72°C for 1 minute first cycle	Variable (Polymorphic	[23]
Coa R	5'-GCT TCC GAT TGT TCG ATG C-3'	repeated for 35 cycle final extension	gene ranged from	
		at 72 °C for 7 minutes	400-800 bp)	
SAEA-F	CCTTTGGAAACGGTTAAAACG	Initial denaturation 93°C for 3 minutes,	127	[15]
SAEA-R	TCTGAACCTTCCCATCAAAAAC	then followed by Denaturation at 92 °C for		
		1 minute Anneal. at 58°C for 1 minute		
SAEB-F	TCGCATCAAACTGACAAACG	Extension at 72°C for 1 minute	477	[15]
SAEB-R	GCAGGTACTCTATAAGTGCC	First cycle repeated for 35 cycle		
		Final extension at 72°C for 7 minutes		

Total cellular DNA was extracted from 0.5 ml TE with QIAGEN DNA extraction kit (Qiagen, Germany) according to manufacturer's protocol for gram-positive bacteria. From each sample, 5 µl of total cellular DNA were then evaluated by PCR with primers and cycling conditions previously described by Zecconi *et al.* [19]. The isolates were tested by a two multiplex PCR assays one of them designed to detect 16SrRNA gene [22] and coagulas (coa) gene by PCR according to Hookey *et al.* [23]. The second multiplex PCR were designed to clarify presence of entertoxins SAE type A and B the primer designed according to Becker *et al.* [15], detailed of primer sequence for each tested gene, specified reaction conditions, product size and corresponding authors were mentioned in Table 1.

PCR product from each reaction (10 µl) was separated on 1.5% agarose gel, stained with ethidium bromide and visualized using an ultraviolet trans-illuminator and photographed. A visible band of appropriate size was considered as a positive reaction. Positive control (all the components for the PCR with *S. aureus* reference strain DNA) and negative control (all the necessary components for the PCR except template DNA) were included in each set of amplifications [25].

RESULTS

On screening milk samples described in Table 2, by using California mastitis test (CMT) and Somatic cell count (SSC), revealed that subclinical mastitis was detected in a prevalence rate of 39.7 and 44.8%, respectively, while on assessing the state of microbiology of tested milk sample a prevalence rate of 34.1% indicating presence of *S. aureus*. While on testing the swabs collected from contact dairy works, a prevalence rate of 44.2 % was detected.

Evaluation of coagulase and Thermo-nuclease activities of isolated *S. aureus* from both milk and contact dairy worker samples in Table 3 revealed presence of coagulase activities in 33.55 and 56.52 %, respectively, while Thermo-nuclease activities were detected in 32.06 and 52.17 %, respectively.

Molecular assessments of *S. aureus* by using multiplex PCR assay (Figure 1) including 16SrRNA and coagulas (coa) genes revealed a successfully amplified product in 49.61 and 82.6% of tested samples of *S. aureus* with 16Sr-RNA gene for milk and dairy workers isolates, respectively. While on detection the prevalence rate of coagulase (Coa) gene on the tested *S. aureus* isolates revealed presence of Coa gene in 40.45 and 69.56% of *S. aureus* with Coa gene for both milk and contact dairy workers isolates, in the same sequence, respectively.

Molecular evaluation of *S. aureus* enterotoxin A and B by multiplex PCR assay, (Table 4 and Figure 2) for both milk and contact dairy workers isolates revealed presence of SEA in 21.37 and 43.47% of *S. aureus* with SEA gene for both milk and contact dairy workers isolates, respectively; while, SEB gene were found in 20.61 and 34.78% of *S. aureus* with SEB gene for both milk and dairy workers isolates, respectively.

Table 2: Screening of collected milk samples by using CMT, SCC and Staphylococcus isolation from milk from dairy worker samples

						Isolation*			
	CMT		SCC (×1000 cells/ml)			Milk		Contact Dairy worker samples	
Test	Cases	%	Cases	%	Mean ± SD	Pos	%	Pos	%
Positive	156	39.7	172	44.8	903.7±0.11	131	34.1	23	44.2
Negative	228	60.3	212	55.2	212±0.021	253	65.9	29	55.8

*Isolation considers positive based on colony morphology on specific media, microscopic appearance by Gram stain and Catalase activities

Table 3: Detection of coagulase and Thermo-nuclease activities of isolated Staphylococcus

	Isolates from milk samples (131)		Isolates from contact dairy workers samples (23)	
Test	Positive	Negative	Positive	Negative
Coagulase test	44 (33.55 %)	87 (66.45%)	13 (56.52 %)	10 (43.48 %)
Thermonuclease	42 (32.06 %)	89 (67.94%)	12 (52.17 %)	11 (47.83 %)

Table 4: Genetic profiling of *S. aureus with Multiples* PCR assays *for* 16SrRNA gene, *coagulase gene SEA gene and SEB for* both milk and dairy workers isolates

	Milk samples (131)		Contact Dairy workers samples (23)	
PCR test	Positive test No. (%)	Negative test No. (%)	Positive test No. (%)	Negative test No. (%)
16SrRNA gene	65 (49.61 %)	66 (50.39%)	19 (82.6 %)	4 (17.4 %)
Coagulas (coa) gene	53 (40.45%)	78 (59.55%)	16 (69.56%)	7 (30.44%)
SEA	28(21.37%)	103 (78.63%)	10 (43.47%)	13 (56.53%)
SEB	27 (20.61%)	104 (79.39%)	8 (34.78%)	15 (65.22%)

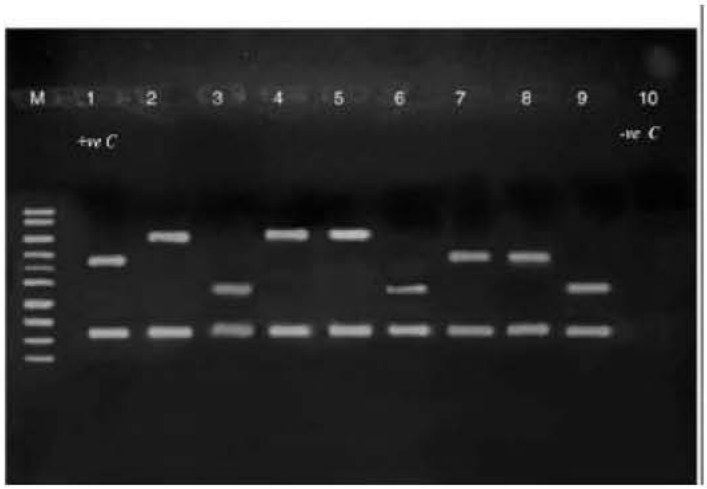

Fig. 1: Electrophoretic pattern of Multiplex PCR for 16SrRNA gene and coagulase (coa) gene where lane M100 bp molecular weight marker while lane No (1) positive control showed positive amplification of *S. aureus* 16SrRNA and Coa gene. Lanes No (2, 3, 4 & 5) showed positive amplification of *S. aureus* 16SrRNA and Coa gene of milk isolates. Lanes No (6, 7, 8 & 9) showed positive amplification of *S. aureus* 16SrRNA and Coa gene of human isolates. Lane No (10) negative control.

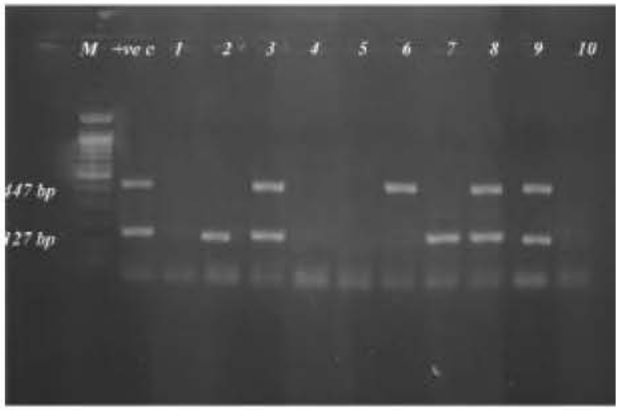

Fig. 2: Electrophoretic pattern of Multiplex PCR for SEA and SEB gene were lane M100 bp molecular weight marker while lanes +ve C ,positive control for enterotoxins lanes No 3, 8&9 positive for SEA and SEB, where lane No (3) from milk while lanes No (8&9) from human isolates. Lanes No 2&7 were positive for SEA from milk human isolates respectively. Lane No (6) were positive for SEB from human samples, lane No (1, 4 & 5) were negative for both toxins

DISCUSSION

Subclinical mastitis means that, although there are no visible or palpable external changes, the infection is present and the inflammation is occurred in the udder [26]. Subclinical mastitis leads to undesirable effect on milk constituents and its nutritional value [3]. Economical losses are due to discarded abnormal milk and milk withheld from cows treated with antibiotic, costs of drugs, veterinary services and increased labor costs. In addition, many problems will be present due to antibiotic residues in human foods, milk and subsequently affect the dairy manufactures, nutritional quality of milk and degrading of milk supplies due to high bacterial or somatic cell count [27]. Regarding public health, mastitis is considered of vital importance due to its association with many zoonotic diseases in which milk acts as a source of infection [18].

Testing the collected quarter milk samples, SCC is found to be more sensitive indicator for detection of subclinical mastitis than CMT. But, CMT remains a quit sufficient reliable test to be applied during milking process. So combining both CMT and SCC are found to be essential to have a reliable screening data for milking animals.

The rate of detection of SCC was higher in subclinical mastitis (927.4±0.12 x 1000 cell/ml milk) when compared with normal cases (229.2±0.39 x 1000 cell/ml milk). On the light of individual count data and isolation assays it is found that most cases suffered from *S. aureus* infection showed higher SCC than 500000 cell/ ml milk. Similar results were described by Awad and Abdel all [28], Abou-Zaid and Bahout [29] and Giraudo *et al.* [30] who recorded the mean of SCC in bacteriologically negative milk samples collected from cows as 236000 and 242000 cells/ml, respectively. While Abou-Zaid and Bahout [29] recorded a mean of 551000 cells/ml for infected samples collected from cows. So, the critical threshold selected for SCC was 240000 cells/ml milk according to Awad [31].

S. aureus is an important cause of mastitis in dairy cows. Infected cows' udders are the main reservoir from which *S. aureus* is transmitted to other cows in the herd and prevention of pathogen transmission from cow to cow reduces the incidence of mastitis [32]. However, when mastitis control measures are implemented, new infections continue to occur and eradication *of S. aureus* intra-mammary infection is difficult to achieve. Infections that originate from sources outside of the mammary gland may contribute to the infection control problem [33]. Many sources of *S. aureus* exist, including housing materials and fodder, equipment and air, bovine skin, non-bovine animals and humans. Teat skin has been suggested as an important reservoir for intra-mammary infection [33], while human-to-bovine transmission has also been proposed. Because many strains of *S. aureus* exist, isolates must be typed to the subspecies level to pinpoint the sources and routes of spread of the pathogen in a population [34].

S. aureus strains have an opportunity to multiply inducing certain problems of public health significance among consumers of dairy products [36,37].

Regarding to obtained data *S. aureus* was found to be of higher prevalence in human samples than in animal's samples, Higher isolation rate was found in human

samples reaching 44.2% (23 out of 52) while in animals a rate of 34.1% (131 out of 384) was found in the same manner, both enzymatic activity of coagulase and thermo-nucleases were found to be of higher incidence rate in human samples than in milk samples.

Using both 16 Sr- RNA and Coa gene for rapid detection of *S. aureus* revealed that a successful amplification of large numbers of tested isolates of human origin (19 out of 23; 82.6%) than those originated from milk (65 out of 131; 49.61%) but the coagulase positive *S. aureus* were higher in human isolates (16 out of 23; 69.56%) than in milk isolates (53 out of 131; 40.45%). The tested coagulase gene revealed presence of 3 polymorphic coagulase amplicons of molecular weight 480,640 and 840 bp by using Coa gene amplification assay. A rapid, easy PCR assay for the detection of *S. aureus* to replace the time-consuming culture-based classical techniques was described by Letertre *et al.* [8]. They found that PCR assay is, sensitive and specific and therefore constitute a very valuable tools for routine applications. Specific primers for PCR detection of *S. aureus* have been directed to 16SrRNA gene [24] and coagulase Coa gene [13, 23]. They proved PCR as rapid, specific, sensitive and reliable methods when compared conventional assays. Both authors [23, 13] described the presence of polymorphic pattern of coagulase gene in *S. aureus* isolates with similar coagulase activities.

Staphylococcal food poisoning is due to the ingestion of food containing one or more preformed enterotoxins (SE) produced by *S. aureus* [4, 38]. The disease is characterized by nausea, vomiting, abdominal cramps and diarrhea lasting from 24 to 48 hours and the complete recovery usually occurs within 1-3 days. It is difficult to obtain accurate estimates of the incidence of *S. aureus* intoxications because most cases are not reported. In spite of being a mild, self-limited illness with low mortality rate, it is considered as one of the most economically important diseases worldwide. Food safety standards include the surveillance of *S. aureus* and according to food type, different contamination levels have been established. Routine detection of *S. aureus* in food is usually carried out by traditional methods based on the use of selective media, like Baird-Parker or RPF agar, for direct enumeration or the recovery of isolates after enrichment in selective broth for 24-48 hours at 37°C [13], Following, suspicious colonies that are positive for DNase and coagulase production, should be identified by biochemical tests. This conventional methodology takes from 5 to 6 days, even when rapid commercial systems are used for identification. Furthermore, they frequently lead to ambiguous results due to doubtful responses of field isolates in some tests.

SEA is the most common enterotoxin recovered from food poisoning outbreaks [4] and it is known that 59% of staphylococcal food poisoning outbreaks are caused by SEA to SEE [15, 40].

On examining the tested isolates for potential production of entererotoxines type A and B (SEA and SEB) using multiplex reaction results revealed a positive amplification assay in revealed presence of SEA in 21.37 and 43.47% of *S. aureus* with SEA gene for both milk and contact dairy workers isolates while SEB gene were found in 20.61 and 34.78% of *S. aureus* with SEB gene for both milk and contact dairy workers isolates. Higher rate of potential entertoxine *S. a*ureus producers was found to be in human isolates than in those recovered from milk samples that denoting that the risk of food contamination is represented by both human and animal sources of *S. aureus*. Similar remarks were mentioned by [8, 13, 15].

In conclusion, testing milk for subclinical mastitis detection should be applied as an essential part of daily evaluation record of each dairy farm, CMT was found to rapid and reliable. SCC data gave an essential data to detect subclinical mastitis cases. Detection of *S. aureus* was found to be an important evaluation point as it constitutes a valuable point for both animal health and public health for contact workers in dairy farms, detection of *S. aureus* by PCR wasfound to be rapid, sensitive and accurate. Special concern should be directed to elucidate the kinetic transfer of *S. aureus* between animals and human. Detection of *S. aureus* entertoxin A and B were found to be easy and rapid by using PCR.

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