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Genotypes and Virulence Factors of *Staphylococcus aureus* Isolated from Bovine Subclinical Mastitis

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Abstract: A total of 300 Egyptian dairy animals (150 cows and 150 buffaloes) were included in a field survey to identify subclinical mastitis (SCM) using the California Mastitis Test (CMT) with individual quarter milk samples (*n = 1154*). Subsequently, samples were screened for the presence of *S. aureus* using classical bacteriological method and PCR. SCM prevalence was high; 42.9%in cows and 36.7%in buffaloes. A (+++) positive CMT-signal (severity of the attack per quarter) was recorded in 35.1% of cows and in 22.9% of buffaloes. *S. aureus* was detected in 24.9 and 14.5% of the CMT-positive cow and buffalo samples, respectively. PCR analysis for 16s–23s ISR rRNA gene, specific to *S. aureus,* showed that 52.5 and 74.2% were positive in the CMT-positive cow and buffalo samples, respectively. The hemolysin type A (*hlA*) and hemolysin type B (*hlB*) virulence genes were found in 10 isolates, whereas Shock Syndrome Toxin-1 (*tst*) and enterotoxins N, O and P (*SEN, O* and *P*) virulence genes were not found in any isolate. This study provides detailed data pertaining to the ecology of virulence in a variety of *S. aureus* causing SCM in Egyptian dairy bovine. It offers the basis for further phenotypic and molecular characterization of *S. aureus* isolates found in raw milk to guarantee safe consumption of raw milk and milk products.

Key words: Subclinical Mastitis · *S. aureus* · Virulence Genes and PCR

INTRODUCTION

Staphylococcal mastitis is a major concern in dairy farming and serious source of subclinical and clinical intra-mammary infections in dairy cows leading to severe worldwide economic losses to the dairy industry [1]. Naturally, *S. aureus* isolates are inhabitants of mucous epithelia and skin of human, dairy cattle and other mammals and spread by virtue of milker'shand/milking machines [2]. Furthermore, *S. aureus* is one of the most important reasons for bacterial food poisoning. From 1991 to 2005, *S. aureus* poisoning cases counted about 15% of the total bacterial induced food-poisoning cases [3]. Milk and milk products are common vehicles of *S. aureus* food poisoning [4].

The pathogenic potential of *S. aureus* depends on numerous cell surface virulence factors and it has capability of producing a variety of toxins [5], extracellular toxins with super antigenic properties, namely enterotoxins A-E, G-K, M-O and Q, exfoliative toxins A and B as well as toxic shock syndrome toxin [3]. The importance to evaluate *S. aureus* pathogenic activity assessing the combination of virulence genes has been emphasized both in human and veterinary medicine [6]. The genotype of *S. aureus* affects its prevalence and the number of infected quarters within a herd [7]. To date, there is limited information about the prevalence of recently identified toxin and exotoxin genes in *S. aureus* associated with infectious diseases in animals and human food poisoning in Egypt. The information about the genetic variability of different *S. aureus* populations would help in the design of efficient therapeutic approaches and improvement of control measure. Therefore, this study was planned to genotype and characterizes *S. aureus* recovered from SCM Egyptian dairy cows and buffaloes using uniplex and multiplex PCR.

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Table 1: Primer sequences, target genes and cycling profiles of PCR assays used in this study.

Primers	Sequence (5'–3')		PCR program	Size (bp)
16-23s rRNA	Forward: TTCGTACCAGCCAGAGGTGGA	35 cycles	95ºC/45s	229
	Reverse: TCTTCAGCGCATCACCAATGCC		50ºC/60s	
			72ºC/30s	
tst	Forward: ATGGCAGCATCAGCTTGATA	30 cycles	94ºC/2m	350
	Reverse: TTTCCAATAACCACCCGTTT		55ºC/2m	
			72ºC/1m	
hlA	Forward: GGT TTA GCC TGG CCT TC	30 cycles	94ºC/45s	550
	Reverse: CAT CAC GAA CTC GTT CG		50ºC/45s	
			72ºC/60s	
hlB	Forward: GCC AAA GCC GAA TCT AAG	35 cycles	94ºC/45s	850
	Reverse: CGC ATA TAC ATC CCA TGG C		57ºC/60s	
			72ºC/80s	
SEN	Forward: CTTCTTGTTGGACACCATCTT	35 cycles	94 °C/30 s	135
	Reverse: GAAATAAATGTGTAGGCTT		55 °C/30 s	
			72 °C/30 s	
SEO	Forward: AAATTCAGCAGATATTCCAT	35 cycles	94 °C/30 s	172
	Reverse: TTTGTGTAAGAAGTCAAGTGTAG		56 °C/30 s	
			72 °C/30 s	
SEP	Forward: ATCATAACCAACCGAATCAC	35 cycles	94 °C/30 s	148
	Reverse: AGAAGTAACTGTTCAGGAGCTA		55 °C/30 s	
			72 °C/30 s	

All PCR programs started with initial denaturation at 94°C (except for *16-23s rRNA* which was 95°C) for 4 minutes and final extension at 72°C for 10 minutes.

MATERIALS AND METHODS

Animals and Sampling: This study was carried out on apparently healthy 300 lactating cows and buffaloes in Kafr El-Sheikh governorate, Egypt during January to September 2016. Milk samples were aseptically collected from apparently healthy udder of hand milking animals with individual quarter milk samples (*n = 1154*). No antimicrobial drugs were administrated to these animals for at least 1 month before samples collection.

California Mastitis Test (CMT): Quarter milk samples were screened in the field using the California Mastitis Test (CMT) [8]. Before sample collection, the udders were thoroughly disinfected with 70% ethanol and dried. The first strips were discarded and a milk sample from each quarter was tested by CMT.CMT-positive quarters' milk samples were collected under aseptic conditions in labeled sterile screw caped bottles and kept at 4ºC for further lab diagnosis.

Isolation and Biochemical Identification of *S. aureus*: Ten milliliters of the milk samples were centrifuged. The sediment was suspended with equal volumes of sterile distilled water. A loopful from each of the prepared milk samples was streaked on Mannitol salt agar and incubated at 37 °C for 48 h. Suspected colonies were described for their morphological characteristic appearance and hemolytic activity, followed by Gram staining before being transferred into slope agar to be subjected for further identification according to Quinn *et al*. [9].

Molecular Identification by PCR: Chromosomal DNA was extracted using a rapid boiling procedure according to Reischl *et al.* [10]. Briefly, 1ml broth per each isolate was taken from the nutrient broth and centrifuged at 5000 rpm to sediment the bacterial pellet. The latter was washed twice using Tris EDTA buffer and finally suspended in 200 µl of lysis buffer [1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. After boiling for 10 min, the suspension was centrifuged for 5 min to sediment bacterial debris. The supernatant was aspirated and from which 5 µl was used directly for PCR amplification.

PCR assays targeting *16-23srRNA*, *tst*, *hlA*, *hlB, SEN, SEO* and *SEP* genes were performed. All assays were performed using total volume of 25µl containing 5µl of template DNA, 20 pmol of each primer (Metabion international AG, Germany) and 1X of PCR mix (PCR Master Mix, Fermentas, Life Science). The PCR cycles were carried out in Eppendorf AG (22331 Hamburg) thermocycler. Detailed sequences of primers and cycling protocols were depicted in Table (1). PCR products were electrophoresed in 1.5% agarose gel containing 0.5X TBE at 100 volts for 40 min. Gels were stained by ethidium bromide and visualized by UV trans-illuminator.

RESULTS AND DISCUSSION

The fact that bovine mastitis covers approximately 30% of all cows' diseases provides evidence for the potential of its economic significance in dairy cattle industry [11]. CMT not only provides a snap-shot in time of the udder health situation of a herd, but also provides a very effective means of plotting of identifying infection trends [12]. In the present study, CMT showed that 459 out of 1154 (39.8%) positive quarters with variable degrees. The degree was related to the CMT score (Table 2). It was found that 245/571 (42.9%) of cows' and 214/583 (36.7%) of buffaloes' quarters were positive. The highest intensity of CMT reaction (39.6 and 40.2%) lies within the scores 2 and 1 for cows and buffaloes, respectively. This is in agreement with Lamey *et al.* [13]. However, lower finding was recorded by Abdel-Rady and Sayed [14]. Despite susceptibility to mastitis is low in buffaloes when compared to cattle [15] the poor management conditions practiced by small buffalo holders in rural areas may anticipate in increased percentage of subclinical mastitis. Health status of mammary gland in milking animals contributes greatly in the economic importance of the farm animals.

Among several bacterial pathogens that can cause mastitis, *S. aureus* is probably the most lethal agent because it causes chronic and deep infection in the mammary glands that is extremely difficult to be cured [16]. Based on biochemical identification, *S. aureus* was isolated from 24.9 and 14.5% of the positive CMT cows and buffaloes samples, respectively (Table 3). This came to some extent with Gonzalo *et al*. [17] and Abd El-Razik *et al*. [18] who reported high incidence of *S. aureus* isolated from milk samples of cows and buffaloes suffering from SCM. Higher findings were also recorded by Abdel-Rady & Sayed [14] and Abdeen *et al.* [19] while lower incidence was reported by Enany *et al.* [20]. *S. aureus* is one of the most prevalent bacteria in subclinical mastitis in dairy cows and in our investigation may be return to milker hands which consider the main tool in distribution of microorganisms from teat to teat and from cow to cow, in addition to lack of hygiene [2].

Molecular identification using *S. aureus* specific gene*16S–23S ISR rRNA* (229 bp) revealed 52.5% (32/61) and 74.2% (23/31) positive isolates (Table 3). The benefit of handling *16S-23S rRNA* gene was the high specificity for detecting *S. aureus* isolates as no other non-specific amplicon was seen. In consistence, amplification of the *16S-23S rRNA* gene was used for quick genotyping numerous *S. aureus* isolates obtained from bovine herds in Switzerland [7]. Moreover, this primer was used to specifically detect staphylococci in multiplex [21] or uniplex [22] PCR assays. Unlike *16S-23S rRNA* gene, previous studies have indicated that the *16S rRNA* gene is not specific enough and cannot differentiate closely related species, as they share high-level (Up to99%) homology in the *16S rRNA* gene sequence [23, 24]. Compared with culture, PCR is less time consuming. It takes less than 24 h to complete, while identification of bacteria by conventional microbiological and biochemical methods requires more than 72 h. Therefore, PCR assay could be used as an alternative method in routine diagnosis of SCM for rapid, sensitive and specific simultaneous detection of *S. aureus* in milk samples [7].

Table 2: Relation between positive CMT and degree of quarter attack.

				CMT categories							
				Total positive CMT		CMT 1		CMT 2		CMT 3	
Animal Spp.	Number of Animals	Blind Quarters	Examined Quarters	No	%	No	%	No	%	No	%
Cow	150	29	571	245	42.9	62	25.3	97	39.6	86	35.1
Buffaloes	150	17	583	214	36.7	86	40.2	79	36.9	49	22.9
Total	300	46	1154	459	39.8	148	32.2	176	38.3	135	29.4

Table 3: Incidence of *S. aureus* isolated from positive CMT of milk samples based on biochemical and PCR tests

	Total positive CMT Quarters		isolated *S. aureus* based on biochemical tests		Molecular identification of *S. aureus* isolates	
Animal Spp.	No.	%	No.	%	No.	%
Cows	245	42.9	61	24.9	32/61	52.5
buff	214	36.7	31	14.5	23/31	74.2
Total	459	39.8	92	20.0	55/92	59.8

Table 4: Molecular identification *S. aureus* isolates and its virulence genes

			Cow S. aureus strains		*Buffalo* S. aureus strains	
Target of *S. aureus*	Gene	PCR product size (bp)	No	%	No	%
16s–23s ISR rRNA	*16s–23s rRNA*	229	32 (32/61)*	52.2	23 (23/31)	74.2
Toxic shock (TSST)	*tst*	*350*	0	0	0	0
Haemolysin type A	*hlA*	550	6 (6/32)**	18.8	3	13.0
Haemolysin type B	*hlB*	840	1 (1/32)**	3.1	0	0
Staph enterotoxin N, O and P	*SE N, O* and *P*	135, 172 and 148	0	0	0	0

Unlike other epidemiologic studies which focused on relationship between severity of mastitis and the virulence factors produced by *S. aureus* [25] in the current study all isolates were negative to TSST-1gene. Orwin *et al.* [26] suggested that *S. aureus* express multiple toxins and that all could contribute to staphylococcal diseases related to super antigenic and emetic toxin, such as food-poisoning and toxic shock syndrome (TSS). In Taiwan, Tsen *et al.* [27] have surveyed the distribution of classical SEs and TSST-1 genotypes in *S. aureus* isolates from food samples. Chiang *et al.* [3] recorded tsst-1 gene, as high as 59.1% of the examined strains, which is higher than that reported by Omoe *et al.* [28]. *S. aureus* may produce one or more of a family of staphylococcal enterotoxins (SEs) [29]. Five classical enterotoxin types, i.e., SEA through SEE and many new types of SEs or super antigens (SAgs), i.e., SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER and SEU have been reported [30]. In the current study, no isolates were positive for the enterotoxins, *N, O and P* genes. Nearly similar result was reported by Memon *et al*. [1] and Kumar *et al*. [31]. However, reports regarding the incidence of isolation of *S. aureus* strains with SEN, SEO, SEP, SEQ, SER and SEU genes in food-poisoning cases are limited. Recently, a comprehensive analysis for 18 classical and newly described staphylococcal super antigenic toxin genes for 67 food-poisoning isolates and 97 healthy human nasal swab isolates has been reported in Japan [28]. The new enterotoxin SEN, SEO [32] and SEP [28] producing strains may play some roles in staphylococcal food-poisoning cases and clinical syndromes.

Molecular investigation of virulence factors revealed presence of *hla* gene (550bp) in 9 strains [6 (18.8%) of cows and 3 (13.0%) of buffaloes] and *hlb* gene (840bp) in 1 (3.1%) of cows isolates (Table 4). This prevalence is less than 85 and 71% reported by Memon *et al.*[1]. The more frequent detection of *hla* than *hlb* in our study comes in agreement with the previous studies of Siti *et al.* [33] and El- Sissi *et al.* [34]. They are able to damage host cells by virtue of their cytolytic effects [31]. The presence of the bacterium is likely to be of little consequence for the most part, but if the defense mechanisms of the host fail, it may become pathogenic.

CONCLUSION

Multiplex PCR is considered as an alternative method for rapid identification of *S. aureus* causing SCM in the dairy animals. This study reported high prevalence of *S. aureus* causing SCM. Therefore, special attention should be given to SCM cases, which act as invisible potential reservoir of virulent *S. aureus* that may constitute a public health hazard.

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REFERENCES

1. Memon, J., Y. Yongchun, K. Jam, Y. Muhammad, B. Rehana, S. Jamila, L. Wang and H. Fan, 2013. Genotypes, Virulence Factors and Antimicrobial Resistance Genes of Staphylococcus aureus isolated in Bovine Subclinical Mastitis from Eastern China. Pak. Vet. J., 33(4): 486-491.
2. Chu, C., C. Yu, Y. Lee and Y. Su, 2012. Genetically divergent methicillin resistant Staphylococcus aureus and sec-dependent mastitis of dairy goats in Taiwan. BMC Vet. Res., 8: 39-46.
3. Chiang, Y.C., W.L. Wan, M.F. Chin, Y.P. Wan, S.C. Chien and Y.T. Hau, 2008. PCR detection of Staphylococcal enterotoxins (SEs) N, O, P, Q, R, U and survey of SE types in Staphylococcus aureus isolates from food-poisoning cases in Taiwan. International Journal of Food Microbiology, 121: 66-73.

4. Jorgensen, H.J., T. Mork, H.R. Hogasen and L.M. Rorvik, 2005. Enterotoxigenic staphylococcus aureus in bulk milk in Norway. Journal of Applied Microbiology, 99: 158-66.
5. Dewanand, R.K., S. Yuvaraj, V.K. Nitin, K.C. Kapil and B.B. Sukhadeo, 2007. PCR-based detection of genes encoding virulence determinants in Staphylococcus aureus from bovine subclinical mastitis cases. J. Vet. Sci., 8(2): 151-154.
6. Piccinini, R., L. Cesaris, V. Dapra, V. Borromeo, C. Picozzi, C. Secchi and A. Zecconi, 2008. The role of teat skin contamination in the epidemiology of staphylococcus aureus intramammary infections. Journal of Dairy Research, 76: 36-41.
7. Fournier, C., P. Kuhnert, J. Frey, R. Miserez, M. Kirchhofer, T. Kaufmann, A. Steiner and H.U. Graber, 2008. Bovine Staphylococcus aureus: Association of virulence genes, genotypes and clinical outcome. Res. Vet. Sci., 85: 439-448.
8. Schalm, O.W., C. Carrol and N.C. Jain, 1971. Bovine mastitis. 1st ed. Lea and Febiger, Philadelphia, USA, pp: 381-395.
9. Quinn, P.J., B.K. Markey, F.C. Leonard, E.S. Fitzpatrick, S. Fanning and P.J. Hartigan, 2011. Veterinary microbiology and microbial disease. 2nd ed. Wiley-Blackwell, j Wiley and Sons ltd publication, UK, pp: 215-242.
10. Reischl, U., M. Pluz, W. Ehret and H. Wolf, 1994. PCR-based detection of mycobacteria in sputum samples using a simple and reliable DNA extraction protocol. Bio. Techniques., 17: 844-845.
11. De Graves, F.J. and J. Fetrow, 1993. Economics of mastitis and mastitis control. Veterinary Clinics of North America: Food Animal Practice, 9: 421-434.
12. Pyorala, S., 2003. Indicators of inflammation in the diagnosis of mastitis. Vet. Res., 34(5): 565-78.
13. Lamey, A.E., A.M. Ammar, E.R. Zaki, N. Khairy, B.S. Moshref and M.K. Refai, 2013. Virulence Factors of Escherichia coli Isolated from Recurrent Cases of Clinical and Subclinical Mastitis in Buffaloes. International Journal of Microbiological Research, 4(1): 86-94.
14. Abdel-Rady, A. and M. Sayed, 2009. Epidemiological Studies on Subclinical Mastitis in Dairy cows in Assiut Governorate. Veterinary World, 2(10): 373-380.
15. Uppal, S.K., K.B. Singh, K.S. Roy, D.C. Nauriyal and K.B. Bansal, 1994. Natural defense mechanism against mastitis: A comparative histo-morphology of buffalo and cow teat canal. Buffalo J., 2: 125-131.
16. Momtaz, H., E. Rahimi and E. Tajbakhsh, 2010. Detection of some virulence factors in Staphylococcus aureus isolated from clinical and subclinical bovine mastitis in Iran. Afr. J. Biotech., 9: 3753-3758.
17. Gonzalo, C., A. Ariznabarrta and J.A. Carriedo, 2002. Mammary pathogens and their relationship to somatic cell count and milk yield losses in dairy ewes. J. Dairy Sci., 85: 1460.
18. Abd El-Razik, K.A., K.A. Abdelrahman, Y.F. Ahmed, A.M. Gomaa and H.A. Eldebaky, 2010. Direct Identification of Major Pathogens of the Bubaline Subclinical Mastitis in Egypt using PCR. Journal of American Science, 6(10): 652-660.
19. Abdeen, E., M. Walid, H. Hussein and S. Roshdy, 2015. PCR for detection of virulence and antibiotic resistance genes of coagulase positive staphylococcus aureus from clinical mastitis in Egypt. International J. Basic and Applied Sciences, 4(3): 315-319.
20. Enany, M.E., Hanora A.M. Said and Shimaa M.I. Aly, 2012. Molecular characterization of bacterial species isolated from clinical and subclinical mastitis in cattle. SCVMJ, XVII, 2: 65-73.
21. Pradhan, P., S.M. Gopinath, G.R. Reddy, H.J. Dechamma and V.V.S. Suryanarayana, 2011. Detection of major pathogens in bovine sub-clinical mastitis by multiplex pcr directly from milk samples in presence of an internal control. Indian Journal of Fundamental and Applied Life Sciences, 1(4): 209-218.
22. Sindhu, N., A. Sharma and V.K. Jain, 2010. Diagnosis of staphylococcal mastitis directly from milk of murrah buffaloes and cross bred cows by 16s-23s ribosomal rna intergenic spacer PCR analysis. Israel Journal of Veterinary Medicine, 65(1): 23-26.
23. Nakanishi, S., T. Kuwahara, H. Nakayama, M. Tanaka and Y. Ohnishi, 2005. Rapid species identification and partial strain differentiation of Clostridium butyricum by PCR using 16S-23S rDNA intergenic spacer regions. Microbiol. Immunol., 49: 613-621.
24. Maggi, R.G., B. Chomel, B.C. Hegarty, J. Henn and E.B. Breitschwerdt, 2006. A Bartonella vinsonii berkhoffii typing scheme based upon 16S-23S ITSand Pap31 sequences from dog, coyote, gray fox and human isolates. Mol. Cell. Probes, 20: 128-134.
25. Akineden, O., C. Annemuller, A.A. Hassan, C. Lammler, W. Wolter and M. Zschock, 2001. Toxin genes and other characteristics of staphylococcus aureus isolates from milk of cows with mastitis. Clinical and Vaccine Immunology, 8: 959-64.

26. Orwin, P.M., D.Y. Leung, H.L. Donahue, R.P. Novick and P.M. Schlievert, 2001. Biochemical and biological properties of staphylococcal enterotoxin K. Infect. Immun., 69: 360-366.
27. Tsen, H.Y., G.K. Yu, K.C. Wang, S.J. Wang, M.Y. Chang and L.Y. Lin, 1998. Comparison of the enterotoxigenic types, toxic shock syndrome toxin (TSST-1) strains and antibiotic susceptibility for enterotoxigenic S. aureus strains isolated from food and clinical samples. Food Microbiol., 15(1): 33-41.
28. Omoe, K., D.L. Hu, H. Takahashi-Omoe, A. Nakane and K. Shinagawa, 2005. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in Staphylococcus aureus isolates. FEMS Microbiol. Lett., 246: 191-198.
29. McCormick, J.K., J.M. Yarwood and P.M. Schlievert, 2001. Toxic shock syndrome and bacterial superantigens: an update. Annu. Rev. Microbiol., 55: 77-104.
30. Letertre, C., S. Perelle, F. Dilasser and P. Fach, 2003. Identification of a new putative enterotoxin SEU encoded by the egc cluster of Staphylococcus aureus. J. Appl. Microbiol., 95: 38-43.
31. Kumar, R., B.R. Yadav and R.S. Singh, 2011. Antibiotic resistance and pathogenicity factors in Staphylococcus aureus isolated from mastitic Sahiwal cattle. J. Biosci., 36: 175-188.
32. Becker, K., A.W. Friedrich, G. Peters and C. Von Eiff, 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SElM, SElO and SElN. Mol. Nutr. Food Res., 48: 488-495.
33. Siti, I.O.S., T. Syarifudin, S. Ngalijan, A. Dwi and P. Feny, 2011. Genotypic characterization of Staphylococcus aureus isolated from bovines, humans and food in Indonesia. J. Vet. Sci., 12(4): 353-361.
34. El-Sissi, A.F., A.G. Jehan and T.E. Mosallam, 2015. Detection of some immunological parameters and hemolysine virulence genes for diagnosis of staphylococcal mastitis. Journal of the Egyptian Veterinary Medical Association, 75(3): 339-353.