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Relative distribution of virulence-associated factors among Australian bovine *Staphylococcus aureus* isolates: Potential relevance to development of an effective bovine mastitis vaccine

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Introduction

Staphylococcus aureus is one of the major contagious pathogens causing bovine mastitis worldwide.[1] It causes contagious mastitis resulting either clinical or subclinical mastitis with increase in the number of somatic cell count (SCC) in milk. More than $130 million is lost by the Australian dairy farmers ($A200/cow/year) every year due to poor udder health caused by mastitis resulting in reduction of milk production, increase in treatment costs, veterinary consultation fees, and number of cow culls. There are multiple pathogens that have been found to be associated with bovine mastitis in Australia.[2] While the relative distribution of the different pathogens causing mastitis may differ in different regions and countries, *S. aureus* is one of the most significant contagious bacterial pathogens causing bovine mastitis and is of concern to public health because of its potential for transmission to humans.

Once the organism enters into the mammary gland, it adheres to epithelial lining and defies the host innate immune defenses by variety of virulence factors such as capsule and protein A which interfere with the process of phagocytosis.[3] Once intra-mammary infection is established, damage to the mammary gland epithelial lining is initiated by ulceration and occlusion of lactiferous ducts and alveoli, infiltration of inflammatory cells in the parenchyma.[4]

S. aureus produces a variety of virulence factors which evade the tissue and host immune system and thereby maintain infection. These virulence factors are capsular polysaccharides, cytotoxins, superantigenic enterotoxins and MSCRAMM (microbial surface components recognizing adhesive matrix molecules). A large number of cytotoxins are produced by *S. aureus* which form pores in the cell membrane causing osmotic swelling leading to cell death. These cytotoxins include leukocidins, phenol soluble modulins (PSMs) and cytolysins. The cytolysins of *S. aureus* are α-, β-, γ-, and δ-toxins, of which α-toxin is well characterized for its contribution to biofilm formation and protective potential.[5,6] β-toxin is a sphingomyelinaseC and 95% of *S. aureus* isolates from bovine mastitis cases produce β-toxin[7] which causes damage to epithelial lining of mammary gland. Gamma (γ) and delta (δ) toxins, bicomponent toxins are synergohymenotropic toxins that act through the synergistic activity of 2 non-associated secretory proteins creating lytic pores in host cells including neutrophils and are assembled from the 2 components secreted separately by the organism as water-soluble molecules.[8] Panton-Valentine Leukocidin (PVL) is encoded by 2 contiguous and cotranscribed genes, *LukS*-PV and *LukF*-PV[9] and creates lytic pores in neutrophils, monocytes and macrophages adversely affecting their function. Phenol soluble modulins (PSMs) are the peptides produced by *S. aureus*, which are cytotoxic and proinflammatory agent. Recent finding has demonstrated that it plays a part in the formation of *S. aureus* biofilm.[10] *S. aureus* produces a number of superantigens including enterotoxins (SEs), Toxic Shock Syndrome toxin and exfoliative toxins. Enterotoxins of *S. aureus* include the classical enterotoxins A to E and the recently identified

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and characterized SEG-SEU toxins.[11,12] These antigens are considered as superantigens due to their ability to release inflammatory cytokines from both T cells and macrophages by binding to the surface of MHC class II proteins and T cell receptors. [13-16]

The first step in establishing infection is the initial attachment of *S. aureus* to eukaryotic membrane and extracellular matrix proteins which is followed by colonization and subsequent infection.[17] Colonization is commonly associated with a variety of adherence factors or adhesins which are known as microbial surface component recognizing adhesive matrix molecules (MSCRAMM). There are over 20 different MSCRAMMs identified, which can be expressed in *S. aureus*[18] that mediate attachment to surface proteins of host cells including collagen, elastin, fibrinogen, thrombospondin, fibronectin, bone sialoprotein and laminin.[19] Major adhesins in this group that mediate the initial attachment of *S. aureus* to the bovine mammary gland, providing the first critical step for establishing infection[19] are clumping factors A and B (ClfA and ClfB),[20] collagen adhesion (CNA),[21] bone sialo binding protein (BBP)[22] and the fibronectin binding proteins A and B (FnBPA and FnBPB).[23] Besides these major adhesins, biofilm-associated protein (*bap*) has also been reported to be associated with primary attachment of *S. aureus* to mammary tissue.[24,25] An accessory gene, *agr*, regulates the production of biofilms including detachment of biofilm that helps in virulence and dissemination of *S. aureus* in the mammary gland resulting in persistent bovine mastitis,[26] whereas penicillin resistance of *S. aureus* is mediated by *blaZ* gene.[27]

Variability in the prevalence of virulence factors in *S. aureus* may result in various levels of severity and forms of mastitis in cows.[28] No studies have been carried out in Australia on the virulence factors of *S. aureus* isolated from clinical cases of bovine mastitis. Aim of this study was to determine the relative distribution of different virulent factors of bovine *S. aureus* isolates in Australia including MSCRAMMS and exotoxins using conventional polymerase chain reaction (PCR) and the available serological methods.

Materials and Methods

One hundred and fifty-four (154) fully characterized *S. aureus* strains of Australian origin isolated from clinical cases of mastitis in cows in Victoria and Queensland were generously donated by Professor Margaret Deighton, (RMIT University), Dr. Sharon de Wet (Queensland Biosecurity laboratory) and Dr. Justine Gibson (University of Queensland). ATCC® 13565™, ATCC® 49775™, ATCC® 51651™ and ATCC® 8096™ were used as positive controls for β-hemolysin, PVL, TSST-1 and α-hemolysin, respectively. The *S. aureus* strains representing CP types 1 (strain M), 2 (strain Smith diffuse), 5 (strain Newman), 8 (USA 400 MW2) and a non-capsulated strain (LAC, USA 300) were donated by Professor Gerald Pier (Harvard Medical School, Boston, USA) and were used as positive control for MSCRAMMS and toxin study. The remaining positive controls for MSCRAMM and toxins were used from our laboratory. These isolates were grown on Mueller Hinton (MH) agar, subcultured in nutrient broth supplemented with 1% glucose and stored on cryobeads (Blackaby Diagnostics) or as glycerol (15%) broth stocks at −80°C.

Genomic DNA was extracted from the *S. aureus* isolates using a kit (MO BIO Laboratories, Inc. Carlsbad, CA). The extracted genomic DNA was stored at −20°C until use. PCR was conducted to detect a total of 32 different virulent genes of *S. aureus* from clinical mastitis cases of cows by using primers as reported elsewhere.[29-41] Briefly, the amplification conditions for *tsst-1, clfA, clfB, cna* and *spa* were 95°C for 5 min, 30 cycles of 95°C for 30 sec, Tm (53°C, 55°C, 47°C, 50°C, 51°C for *tsst-1, clfA, clfB, cna* and *spa*) for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10 min. The PCR conditions for *fnbpA, fnbpB, hlb, sdrE, bbp, isdA* and *sdrD* were 95°C for 5 min, 35 cycles of 95°C for 30 sec, Tm (48°C, 56°C, 51°C, 50°C, 53°C, 52°C, 52.3°C for *fnbpA, fnbpB, hlb, sdrE, bbp, isdA* and *sdrD*) for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10min. *isdB* primers were developed in our lab with the amplification conditions at 35 cycles of 95°C for 30 sec, 55°C for 1min and 72°C for 2 min with a final extension of 72°C for 10 min. Amplification conditions of *hla* were 95°C for 5 min, 38 cycles of 95°C for 30 sec, 47°C for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10 min. The cycling conditions for *bap, blaz* and *agr* types (*agr* type I-IV) were 94°C for 5 min, 30 cycles of 94°C for 30 sec, Tm (42°C, 60°C, 60°C for *bap, blaz* and *agr* types) for 30 sec and 72°C for 60 sec with a final extension of 72°C for 10 min. The amplification conditions for *sea, seb, sec, sed, see, seg, seh, sei* and *sej* were 95°C for 5 min, 30 cycles of 95°C for 2 min, Tm (50°C for *sea* to *see*, sei, sej and 48.4°C for *seh* and 50°C for *sei*) for 1 min and 72°C for 1 min with a final extension of 72°C for 5 min. *eta* and *etb* were amplified at 95°C for 5 min, 30 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min with a final extension of 72°C for 10 min. All PCR products were analyzed by agarose gel (1.5%) electrophoresis, staining with 0.8 uL/100 mL of Midori Green DNA Stain (Nippon Genetics) using 1× SB Buffer. An O'RangeRuler DNA Ladder (100–1500 bp, Fermentas) was used for comparing the approximate band sizes after visualizing on UV transilluminator.

Detection of the SEA, SEB, SEC1, SEC2, SEC3, SED and SEE toxins in the 154 strains of *S. aureus* was carried out using 3M™ Tecra™ Staph Enterotoxins Visual Immunoassay or 3M™ Tecra™ SET VIA kits (3 M Australia Pty Ltd). The test was performed according to the instructions of the manufacturer. Briefly, wash solution provided with the kit was used to soak *S. aureus* cells and incubated at room temperature. 200 μL sample was added to the SE pre-coated wells and optical density was read at 405 nm. Samples showing OD ≥ 0.2 were considered positive. Two sets of positive and negative controls provided with the kit were run along with the test samples.

SET-RPLA Toxin Detection kit (Themo-Fisher Scientific, Australia) was used to confirm presence or absence of enterotoxin A–D in 154 *S. aureus* strains of bovine origin. Briefly, bacterial suspension prepared as per the protocol provided with the kit and latex

particles linked to anti-enterotoxin A–D antibodies was added to bacterial suspension. The antigen-antibody suspensions were incubated at room temperature for 24 h and observed for agglutination to confirm the presence of enterotoxins.

Detection of *mecA* and *pvl* gene were accomplished by using GenoType® MRSA assay (Hain-Lifesciences) according to the instructions of the manufacturer. Briefly, 45 μl of amplification mix was prepared by mixing 35 μl PNM, 5 μl 10 × polymerase incubation buffer, 3 μl MgCl2 solution, 1.6 μl nuclease free water and 0.4 μl DNA polymerase. To the mixture 5 μl of genomic DNA was added to make a total volume of 50 μl. The amplification conditions were 95°C for 5 min, 22 cycles each of 95°C for 20 sec and 60°C for 30 sec. The amplified product was stored at −20°C until the next step. The final step was hybridization biotin-labeled amplicons to membrane-bound probes. Evaluation and interpretation of results was done as per the bands developed in the strip and comparing with the guidelines provided with the kit.

Correlation coefficient, represented as Pearson *r* value, between the serological and the genotyping method, for SEA, SEB, SEC and SED positive *S. aureus* strains, were determined using Microsoft Excel, Windows 10.

Results and Discussion

Among the MSCRAMM, *clfA, clfB, spa, isdA, isdB, sdrD* and *sdrE* were the predominant antigens detected in the *S. aureus* isolates (**Table 1**). None of the strains were found to be positive for *mecA* or *agr IV* gene. On the other hand, among the toxins, α-toxin and β-toxin were the most prevalent cytotoxins encoded and/or produced by clinical *S. aureus* isolates from bovine mastitis cases in Australia, followed by *seh, sec, seg* and *sei*. Bramley et al. (1989) investigated the putative role of α-toxin and β-toxin in mouse mastitis by constructing single or double mutants of a wild type bovine isolate, that killed majority of the mice within 48 hours post-infection via the mammary gland. However, the mutant strains did not kill mice despite a significantly higher recovery from the mammary gland.[42] The correlation coefficient (*r*) between the serological and genotyping methods for detection of SEA, SEB, SEC and SED positive *S. aureus* isolates, in our study, was determined to be 0.98.

Table 1. Distribution of virulent factor genes in *S. aureus* isolates of bovine mastitis origin in Australia

S. aureus **toxin gene**	**Number of strains positive**	**Percentage from total samples (%)**
cna	48	31.2
clfA	141	91.56
clfB	143	92.86
spa	135	87.7
fnbpA	84	54.5
fnbpB	2	1.3
bbp	14	9.09
isdA	151	98.1
isdB	154	100
sdrD	151	98.1
sdrE	147	95.5
Bap	2	1.3
agrI	79	51.3
agrII	2	1.3
agrIII	46	29.9
agrIV	0	0
blaz	31	20.2
hla	145	94.16
hlb	128	83.12
tsst-1	13	8.44
eta	0	0
etb	0	0
mecA	0	0
pvl	4	2.6
sea	4	2.6
seb	1	0.65
sec	36	23.4
sed	2	1.3
see	1	0.65
seg	27	17.5
seh	50	32.5
sei	20	13
sej	2	1.3

To the best of our knowledge, this is the first study on the prevalence of virulence factors in *S. aureus* isolates of bovine mastitis origin in Australia. However, the prevalence of virulence factors associated with *S. aureus* isolates from bovine mastitis cases in different countries has been reported previously. [43-46] The prevalence of different virulence-associated genes detectable among the *S. aureus* isolates from Finland revealed that majority of the isolates carried haemolysin genes (76.7–97.4%), LukED (96.6%) and at least one gene for pyrogenic toxin superantigen (69.0%).[44] A total of 67.8% of the bovine mastitis *S. aureus* isolates from Japan were reported to harbour *tst, sec, seg* and *sei* gene.[46]. Ikawaty et al (2010) reported the presence of *hlb* in all strains of *S. aureus* isolated from bovine mastitis in Netherland and similar to our findings could not detect *eta* and *etb* genes among the isolates.[28] In contrast to our finding, only 21%, 33% and 18% of the Dutch *S. aureus* strains carried *clf*A, *sdr*E and *cna* genes, respectively. All the Australian bovine *S. aureus* strains harboured the *isd*B gene.

Whether the relative distribution of virulence-associate factors of *S. aureus* is relevant to the development of an effective vaccine against bovine mastitis cannot be deduced from the data presented in this investigation. However, it may be worth considering this information in the development of an effective vaccine against bovine mastitis. An ideal vaccine for the prevention of bovine mastitis should be able to mount immune responses to at least the most prevalent MSCRAMM, immune evading capsular polysaccharides and toxins. Only limited studies have been carried out for the development of effective vaccines for the prevention of *S. aureus*- associated bovine mastitis, with most investigations having dealt with prevention of systemic infections, using predominantly conjugate vaccines, using an invasive mouse model system.[47-48] However, with the exception of killed whole cell and live attenuated vaccines[49] which theoretically represent all the MSCRAMM, major surface-associated polysaccharide antigens and membrane-bound toxins such as α-toxin, all the other types of vaccines, particularly conjugate vaccines, have involved ascertaining the protective efficacy of vaccines using permutation and combination of antibodies raised against a total of up to a maximum of 6 antigens.[47] The vaccine candidates, against which, the above-mentioned antibodies were produced consisted of either PNAG or CP conjugated to different MSCRAMM. Other types of vaccine candidates that have been evaluated for immunogenicity and/or protective potential include chimeric GapC/GapB proteins of *S. aureus*[48] and B cell epitope of ClfA fused with a surface immunogenic protein (Sip) of *Streptococcus agalactiae*.[50] However, no studies on the protective potential of vaccines containing all the highly prevalent virulence-associated antigens of *S. aureus* against bovine mastitis using the mouse mastitis model, have been reported, warranting further investigations.

Conclusion

This study revealed the relative distribution of the detectable virulence factors of *S. aureus* isolated from clinical bovine mastitis cases in Australia, highlighting those whose function may need to be neutralized promoting the discovery of novel delivery strategies for the development of an effective vaccine against *S. aureus*–associated bovine mastitis in Australia.

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References

1. Zecconi A. Contagious mastitis control program: the *Staphylococcus aureus* case. Cattle Pract 2006; 14: 67-76.
2. Gogoi-Tiwari J, Babra C, Tiwari HK, Williams V, Wet SD, Gibson J, Paxman A, Morgan E, Costantino P, Sunagar R, et al. Trends in therapeutic and prevention strategies for management of bovine mastitis: An Overview. J Vaccines Vaccin 2013; 4: 1-11.
3. Nanra JS, Buitrago SM, Crawford S, Ng J, Fink PS, Hawkins J, Scully IL, McNeil LK, Aste-Amezaqa JM, Cooper D, et al. Capsular polysaccharides are an important immune evasion mechanism for *Staphylococcus aureus*. Hum Vaccin Immunother 2013; 9: 480-7; PMID:23249887; http://dx.doi.org/10.4161/hv.23223
4. Paape MJ, Mehrzad J, Zhao X, Detileux J, Burvenich C. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplas 2002; 7: 109-21; http://dx.doi.org/10.1023/A:1020343717817
5. Wadstrom T. Biological effects of cell damaging toxins: Staphylococci and staphylococcal infections. Adlam C, and C.S.F. (eds). Academic Press, New York. 1983. 671-704 p.
6. Caiazza NC, O'Toole GA. Aplha toxin is required for biofilm formation by *Staphylococcus aureus*. J Bacteriol 2003; 185: 3214-17; PMID:12730182; http://dx.doi.org/10.1128/JB.185.10.3214-3217.2003
7. Tollersrud T. *Staphylococcus aureus* mastitis: Bacterial characteristics and host immune responses. Ph.D thesis, 2011, Natl Vet Instt. Oslo, Norway.
8. Hirsh DC, Maclachlan NJ, Walker R. Veterinary Microbiology. 2nd Ed. Blackwell Publishing Company, New Jersey, USA, 2004.
9. Rainard P, Corrales JC, Barrio MB, Cochard T, Poutrel B. Leucotoxic activities of *Staphylococcus aureus* strains isolated from cows, ewes and goats with mastitis: Importance of LukM/LukF'-PV leukotoxin. Clin Diagn Lab Immunol 2003; 10: 272-7; PMID:12626454

10. Periasamy S, Chatterjee SS, Cheung GYC, Otto M. Phenol soluble modulins in Staphylococci: what are they originally for? Commun Integr Biol 2012; 5: 275-7; PMID:22896791; http://dx.doi.org/10.4161/cib.19420
11. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of *Staphylococcus aureus*. Clin Microbiol Rev 2000; 13: 16-34; PMID:10627489; http://dx.doi.org/10.1128/CMR.13.1.16-34.2000
12. Smyth DS, Hartigan PJ, Meaney WJ, Fitzgerald JR, Deobald CF, Bohach GA, Smyth CJ. Superantigen genes encoded by the *egc* cluster and SaPIbov are predominant among *Staphylococcus aureus* isolates from cows, goats, sheep, rabbits and poultry. J Med Microbiol 2005; 54:401-11; PMID:15770028; http://dx.doi.org/10.1099/jmm.0.45863-0
13. Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. Sci 1990; 248: 705-11; http://dx.doi.org/10.1126/science.2185544
14. Balaban N, Rasooly A. Staphylococcal enterotoxins. Int J Food Microbiol 2000; 61: 1-10; PMID:11028954; http://dx.doi.org/10.1016/S0168-1605(00)00377-9
15. Ulrich RG. Evolving superantigens of *Staphylococcus aureus*. FEMS Immunol Med Microbiol 2000; 27: 1-7; PMID:10617783; http://dx.doi.org/10.1111/j.1574-695X.2000.tb01404.x
16. Schlievert PM, Case LC, Strandberg KL, Abrams BB, Leung DYM. Superantigen Profile of *Staphylococcus aureus* Isolates from Patients with Steroid-Resistant Atopic Dermatitis. Clin Infect Dis 2008; 46: 1562-7; PMID:18419342; http://dx.doi.org/10.1086/586746
17. Brouillette E, Talbot BG, Malouin F. The fibronectin binding proteins of *Staphylococcus aureus* may promote mammary gland colonization in a lactating mouse model of mastitis. Infect. Immun 2003; 71: 2292-5; PMID:12654860; http://dx.doi.org/10.1128/IAI.71.4.2292-2295.2003
18. Walsh EJ, Miajlovic H, Gorkun OV, Foster TJ. Identification of the *Staphylococcus aureus* MSCRAMM clumping factor B (ClfB) binding site in the alphaC-domain of human fibrinogen. Microbiol 2008; 154: 550-8; http://dx.doi.org/10.1099/mic.0.2007/010868-0
19. Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. Trends Microbiol 1998; 6: 484-8. Review; PMID:10036727; http://dx.doi.org/10.1016/S0966-842X(98)01400-0
20. McDevitt D, Francois P, Vaudaux P, Foster TJ. Molecular characterization of the clumping factor (Fibrinogen receptor) of Staphylococcus aureus. Mol Microbiol 1994; 11:237-48; PMID:8170386; http://dx.doi.org/10.1111/j.1365-2958.1994.tb00304.x
21. Patti JM, Jonsson H, Guss B, Switalski LM, Wiberg K, Lindberg M, Hook M. Molecular characterization and expression of a gene encoding of *Staphylococcus aureus* collagen adhesion. J Biol Chem 1992; 267:1766-72.
22. Tung H, Guss B, Hellman U, Persson L, Rubin K, Ryden C. A bone sialoprotein binding protein from *Staphylococcus aureus*: a member of the Staphylococcal Sdr family. Biochem J 2000; 345: 611-9; PMID:10642520; http://dx.doi.org/10.1042/0264-6021:3450611
23. O'Neill E, Pozzi C, Houston P, Smyth D, Humphreys H, Robinson DA, Loughman A, Foster TJ, O'Gara J. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronecting-binding proteins, FnBPA and FnBPB. J Bacteriol 2008; 190: 3835-50; PMID:18375547; http://dx.doi.org/10.1128/JB.00167-08
24. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penades JR. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol 2001; 183: 2888-96; PMID:11292810; http://dx.doi.org/10.1128/JB.183.9.2888-2896.2001
25. Cucarella C, Angeles TM, Ubeda C, Pilar TM, Monzon M, Peris C, Amorena B, Lasa, I, Penades JS. Role of biofilm associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. Infect Immunol 2004; 72: 2177-85; http://dx.doi.org/10.1128/IAI.72.4.2177-2185.2004
26. Boles BR, Horswill AR. Staphylococcal biofilm disassembly. Trends Microbiol 2011; 19: 449-55; PMID:21784640; http://dx.doi.org/10.1016/j.tim.2011.06.004
27. Melchior MB, Van Osch MHJ, Lam TJGM, Vernooij JCM, Gaastra W. Extended biofilm susceptibility assay for *Staphylococcus aureus* bovine mastitis isolates: evidence for association between genetic makeup and biofilm susceptibility. J Dairy Sci 2011; 94: 5926-37; PMID:22118083; http://dx.doi.org/10.3168/jds.2011-4243
28. Ikawaty R, Brouwer EC, Duijkeren EVm, Mevius D, verhoef J, Fluit AC. Virulence factors of genotyped bovine mastitis *Staphylococcus aureus* isolates in Netherlands. Int J dairy Sci 2010; 5: 60-70; http://dx.doi.org/10.3923/ijds.2010.60.70
29. Smith K, Gould KA, Ramage G, Gemmell CG, Hinds J, Lang S. Influence of tigecycline on expression of virulence factors in biofilm-associated cells of methicillin resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2010; 54:380-7; PMID:19858261; http://dx.doi.org/10.1128/AAC.00155-09
30. Stutz K, Stephan R, Tasara T. SpA, ClfA, and FnbA genetic variations lead to Staphaurex test-negative phenotypes in bovine mastitis *Staphylococcus aureus* isolates. J Clin Microbiol 2011; 49: 638-46; PMID:21147952; http://dx.doi.org/10.1128/JCM.01148-10
31. Montesinos I, Salido E, Delgado T, Cuervo M, Sierra A. Epidemiologic genotyping of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis at a university hospital and comparison with antibiotyping and protein A and coagulase gene polymorphisms. J Clin Microbiol 2002; 40: 2119-25; PMID:12037075; http://dx.doi.org/10.1128/JCM.40.6.2119-2125.2002
32. Booth MC, Pence LM, Mahasreshti P, Callegan MC, Gilmore MS. Clonal associations among *Staphylococcus aureus* isolates from various sites of infection. Infect Immun 2001; 69: 345-52; PMID:11119523; http://dx.doi.org/10.1128/IAI.69.1.345-352.2001
33. Tristan A, Ying L, Bes M, Etienne J, Vandenesch F, Lina G. Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. J clin Microbiol 2003; 41:4465-7; PMID:12958296; http://dx.doi.org/10.1128/JCM.41.9.4465-4467.2003
34. Verkaik NJ, Boelens HA, de Vogel CP, Tavakol M, Bode LG, Verbrugh HA, van Belkum A, van Wamel WJ. Heterogeneity of the humoral immune response following *Staphylococcus aureus* bacteremia. Eur J Clin Microbiol Infect Dis 2010; 29: 509-18; PMID:20186449; http://dx.doi.org/10.1007/s10096-010-0888-0
35. Sabat A, Melles DC, Martirosian G, Grundmann H, van Belkum A, Hryniewicz W. Distribution of the serine-aspartate repeat protein-encoding sdr genes among nasal-carriage and invasive *Staphylococcus aureus* strains. J Clin Microbiol 2006; 44: 1135-8; PMID:16517913; http://dx.doi.org/10.1128/JCM.44.3.1135-1138.2006
36. Gilot P, Linca G, Cochard T, Poutrel B. Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. J Clin Microbiol 2002; 40:4060-7; PMID:12409375; http://dx.doi.org/10.1128/JCM.40.11.4060-4067.2002
37. Martineau F, Picard FJ, Grenier L, Roy PH, Ouellette M, Bergeron MG. Multiplex PCR assays for the detection of clinically relevant antibiotic resistance genes in staphylococci isolated from patients infected after cardiac surgery. The ESPRIT Trial. J Antimicrob Chemother 2000; 46: 527-34; PMID:11020248; http://dx.doi.org/10.1093/jac/46.4.527
38. Nada HA, Gomaa NI, Elakhras A, Wasfy R, Baker RA. Skin colonization by superantigen-producing *Staphylococcus aureus* in Egyptian patients with atopic dermatitis and its relation to disease severity and serum interleukin-4 level. Int J Infect Dis 2012; 16:e29-33; PMID:22040925; http://dx.doi.org/10.1016/j.ijid.2011.09.014
39. Sauer P, Sila J, Stosova T, Vecerova R, Hejnar P, Vagnerova I, Kolar M, Raclavsky V, Petrzelova J, Loveckova Y, et al. Prevalence of genes encoding extracellular virulence factors among meticillin-resistant *Staphylococcus aureus* isolates from the University Hospital, Olomouc, Czech Republic. J Medical Microbiol 2008; 7: 403-10; http://dx.doi.org/10.1099/jmm.0.47413-0
40. Ramakrishna US, Kingston JJ, Harishchandra Sripathi M, Batra HV. Taguchi optimization of duplex PCR for simultaneous identification of *Staphylococcus aureus* and Clostridium perfringens α toxins. FEMS Microbiol Lett 2013; 340: 93-100; PMID:23278425; http://dx.doi.org/10.1111/1574-6968.12070
41. Rall VL, Vieira FP, Rall R, Vieitis RL, Fernandes A, Jr Candeias JM, Cardoso KF, Araujo JP Jr. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. Vet Microbiol 2008; 132: 408-13; PMID:18572331; http://dx.doi.org/10.1016/j.vetmic.2008.05.011
42. Bramley AJ, Patel AH, O'Reilly M, Foster R, Foster TJ. Roles of α-toxin and β-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. Infect Immun 1989; 57: 2489-94; PMID:2744856
43. Zecconi A, Cesaris L, Liandris B, Dapra V, Piccinini R. Role of several *Staphylococcus aureus* virulence factors on the inflammatory response in bovine mammary gland. Microb Pathog 2006; 40:17783.
44. Haveri M, Roslof A, Rantala L, Pyorala S. Virulence genes of bovine *Staphylococcus aureus* from persistent and nonpersistent intramammary infections with different clinical characteristics. J Appl Microbiol 2007; 103: 993-1000; PMID:17897203; http://dx.doi.org/10.1111/j.1365-2672.2007.03356.x
45. Fournier C, Kuhnert P, Frey J, Miserez R, Kirchhofer M, Kaufmann T, Steiner A, Graber HU. Bovine *Staphylococcus aureus*: Association of virulence genes, genotypes and clinical outcome. Res Vet Sci 2008; 85: 439-48; PMID:18358507; http://dx.doi.org/10.1016/j.rvsc.2008.01.010
46. Katsuda K, Hata E, Kobayashi H, Kohmoto M, Kawashima K, Tsunemitsu H, Eguchi M. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. Vet Microbiol 2005; 105: 301-5; PMID:15708828; http://dx.doi.org/10.1016/j.vetmic.2004.12.004
47. Pozzi C, Wilk K, Lee JC, Gening M, Nifantiev N, Pier GB. Opsonic and Protective Properties of Antibodies Raised to Conjugate Vaccines Targeting Six *Staphylococcus aureus* Antigens. PLoS ONE 2012; 7: e46648; PMID:23077517; http://dx.doi.org/10.1371/journal.pone.0046648
48. Perez-Casal J, Prysliak T, Kerro-Dego O, Potter AA. Immune responses to a *Staphylococcus aureus* GapC/B chimera and its potential use as a component of a vaccine for *S. aureus* mastitis. Vet Immunol Immunopathol 2006; 109: 85-97; PMID:16165220; http://dx.doi.org/10.1016/j.vetimm.2005.07.024
49. Garcia V, Gomez M, Iglesias M, Sanjuan N, Gherardi M, Cerquetti MC, Sordelli D. Intramammary immunisation with live-attenuated *Staphylococcus aureus*: microbiological and immunological studies in a mouse mastitis model. FEMS Immunol Med Microbiol 1996; 14: 45-51; PMID:8804975; http://dx.doi.org/10.1016/0928-8244(96)00020-X
50. Xu H, Hu C, Gong R, Chen Y, Ren N, Xiao G, Xie Q, Zhang M, Liu Q, Guo A, et al. Evaluation of a novel chimeric B cell epitope-based vaccine against mastitis induced by either *Streptococcus agalactiae* or *Staphylococcus aureus* in mice. Clin Vaccine Immunol 2011; 18: 893-900; PMID:21508165; http://dx.doi.org/10.1128/CVI.00066-11