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

Jully Gogoi-Tiwari¹, Charlene Babra Waryah¹, Karina Yui Eto¹, Modiri Tau¹, Kelsi Wells¹, Paul Costantino¹, Harish Kumar Tiwari², Shrikrishna Isloor³, Nagendra Hegde⁴, and Trilochan Mukkur¹,*

¹School of Biomedical Sciences; Faculty of Health Sciences; Curtin Health Innovation Research Institute; CHIRI Biosciences Research Precinct; Curtin University; Perth, Australia; ²School of Veterinary and Life Sciences; Murdoch University; Perth, Australia; ³Veterinary College; Karnataka Veterinary; Animal and Fisheries Sciences University; Hebbal; Bangalore, India; ⁴Ella Foundation; Genome Valley; Shameerpet Mandal; Hyderabad, India

**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.⁵⁶ β-toxin is a sphingomyelinaseC and 95% of *S. aureus* isolates from bovine mastitis cases produce β-toxin⁷ 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...
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. aureus18 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 infection19 are clumping factors A and B (CfA and CfB),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, bap, isdB 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, bap, isdB and sdrD) for 30 sec and 72°C for 45 sec with a final extension of 72°C for 10 min. The PCR conditions for etb, clfA, clfB, cna and etb 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 amplification conditions of bla were 95°C for 5 min, 30 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 hlb, sdrE 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 hlb, sdrE 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 etb, cna and etb were 95°C for 5 min, 30 cycles of 95°C for 30 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 μL/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 \textit{mecA} and \textit{pvl} gene were accomplished by using GenoType\textsuperscript{®} 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 \textit{S. aureus} strains, were determined using Microsoft Excel, Windows 10.

**Results and Discussion**

Among the MSCRAMM, \textit{clfA}, \textit{clfB}, \textit{spa}, \textit{isdA}, \textit{isdB}, \textit{sdrD} and \textit{sdrE} were the predominant antigens detected in the \textit{S. aureus} isolates (Table 1). None of the strains were found to be positive for \textit{mecA} or \textit{agr IV} gene. On the other hand, among the toxins, \( \alpha \)-toxin and \( \beta \)-toxin were the most prevalent cytotoxins encoded and/or produced by clinical \textit{S. aureus} isolates from bovine mastitis cases in Australia, followed by \textit{seh}, \textit{sec}, \textit{seg} and \textit{sei}. Bramley et al. (1989) investigated the putative role of \( \alpha \)-toxin and \( \beta \)-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.\textsuperscript{42} The correlation coefficient \(( r )\) between the serological and genotyping methods for detection of SEA, SEB, SEC and SED positive \textit{S. aureus} isolates, in our study, was determined to be 0.98.

| \textit{S. aureus} toxin gene | Number of strains positive | Percentage from total samples (%) |
|-----------------------------|---------------------------|----------------------------------|
| \textit{cna}                | 48                        | 31.2                             |
| \textit{clfA}               | 141                       | 91.56                            |
| \textit{clfB}               | 143                       | 92.86                            |
| \textit{spa}                | 135                       | 87.7                             |
| \textit{fnbpA}              | 84                        | 54.5                             |
| \textit{fnbpB}              | 2                         | 1.3                              |
| \textit{bbp}                | 14                        | 9.09                             |
| \textit{isdA}               | 151                       | 98.1                             |
| \textit{isdB}               | 154                       | 100                              |
| \textit{sdrD}               | 151                       | 98.1                             |
| \textit{sdrE}               | 147                       | 95.5                             |
| \textit{Bap}                | 2                         | 1.3                              |
| \textit{agrI}               | 79                        | 51.3                             |
| \textit{agrII}              | 2                         | 1.3                              |
| \textit{agrIII}             | 46                        | 29.9                             |
| \textit{agrIV}              | 0                         | 0                                |
| \textit{blaz}               | 31                        | 20.2                             |
| \textit{hla}                | 145                       | 94.16                            |
| \textit{hlb}                | 128                       | 83.12                            |
| \textit{tsst-1}             | 13                        | 8.44                             |
| \textit{eta}                | 0                         | 0                                |
| \textit{etb}                | 0                         | 0                                |
| \textit{mecA}               | 0                         | 0                                |
| \textit{pvl}                | 4                         | 2.6                              |
| \textit{sea}                | 4                         | 2.6                              |
| \textit{seb}                | 1                         | 0.65                             |
| \textit{sec}                | 36                        | 23.4                             |
| \textit{sed}                | 2                         | 1.3                              |
| \textit{see}                | 1                         | 0.65                             |
| \textit{seg}                | 27                        | 17.5                             |
| \textit{seh}                | 50                        | 32.5                             |
| \textit{sei}                | 20                        | 13                               |
| \textit{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 *tgt*, *sec*, *seg* and *sei* gene.\(^{46}\) Ikawaty et al (2010) reported the presence of *hbl* in all strains of *S. aureus* isolated from bovine mastitis in Netherlands and similar to our findings could not detect *eta* and *eth* genes among the isolates.\(^{28}\) In contrast to our finding, only 21%, 33% and 18% of the Dutch *S. aureus* strains carried *clfA*, *sdrE* and *cna* genes, respectively. All the Australian bovine *S. aureus* strains harboured the *isdB* 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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