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Typing of *Staphylococcus aureus* isolated from bovine mastitis cases in Australia and India

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Abstract

Objective To determine the prevalence of the different capsular polysaccharide (CP) and the major surface-associated non-CP antigen 336 (SP-336) types among *S. aureus* isolated from bovine mastitis cases in Australia and India.

Methods A total of 414 strains (154 from Australia and 260 from India) isolated from clinical bovine mastitis were included for the study. Mouse antisera raised against CP types (CP1, CP2, CP5, and CP8) or SP-336 were used in slide agglutination tests and compared to detection of *cap1*, *cap5* and *cap8* gene fragments by PCR.

Results Serological studies revealed the presence of CP2, CP5, CP8 and SP-336 in 9.1%, 23.4%, 31.8%, and 5.8% of the Australian versus 0.8%, 46.9%, 13.1% and 0% of the Indian isolates, respectively. By PCR, CP1, CP5 and CP8 accounted for 0%, 26.6% and 32.4% of the Australian versus 3.9%, 85% and 8.1% of the Indian isolates, respectively.

Conclusions Both PCR and serological study have demonstrated that CP5 and CP8 are the predominant capsular types in Australia whereas CP5 is the predominant capsular type in India. The study has also demonstrated a strong correlation between both the methods of typing for CP1, CP5, CP8 and non-typeable *S. aureus* strains. Prevalence of high percentages of non-typeable isolates in both the countries highlights the importance of continued investigations on the identification of unique surface-associated polysaccharide antigens prevalent among *S. aureus* isolates for the formulation of CP and SP-based vaccines for bovine mastitis.

Key words capsular and polysaccharide antigen 336, dairy cattle, mastitis, serology, molecular typing

Abbreviations CP, Capsular polysaccharide; SP, Surface polysaccharide; PCR, Polymerase chain reaction; MSSA, Methicillin sensitive *Staphylococcus aureus*; AISRF, Australia India
Strategic Research Fund; MH, Mueller Hinton; ATCC, American type culture collection; CFU, Colony forming unit; DNA, Deoxyribonucleic acid; UV, Ultra violet; PNAG, Poly-N-acetyl glucosamine; CHIRI, Curtin Health Innovation Research Institute.

**Introduction**

The Australian and Indian dairy industries sustain economic losses estimated to be more than US$130 million\(^1\) and US$1200 million\(^2\), respectively, due to poor udder health, which is mainly caused by mastitis. *Staphylococcus aureus* is one of the predominant causative agents of clinical mastitis in most countries\(^3\)-\(^7\) including India\(^2,8\) and Australia.\(^9\) The current conservative practice of treating mastitis with antibiotics is not only economically unviable in the long term, especially for the marginal and small-scale dairy farmers in India, but also promotes the emergence of strains resistant to antibiotics including methicillin.\(^10\)-\(^15\) Given the recent reports that even MSSA (methicillin-sensitive *Staphylococcus aureus*) can become resistant to certain antimicrobials when presented as biofilms adds another dimension to bovine mastitis caused even by MSSA.\(^16\)-\(^18\) In addition, there is increasing evidence for cross transmission of virulent *S. aureus* between dairy cattle and humans\(^19\)-\(^23\) with implications for public health. There is an urgent need for a suitable vaccine against mastitis to reduce the bacterial load and the probability of successful cross transmission.

Capsular polysaccharide (CP) is one of the most important virulence factors of *S. aureus*\(^24\),\(^25\) as it confers resistance to phagocytosis\(^26\) and prolongs persistence of the pathogen in the blood stream of the host.\(^27\) Earlier serological studies suggested the existence of 11 different serotypes of CP.\(^28\),\(^29\) However, a more recent study\(^30\) indicated that there were only four types (CP 1, 2, 5 and 8), all the other reported types representing mutated versions. Most human clinical isolates have been reported to express either CP5 or CP8, accounting for ~80% of the
total isolates from all sources. The rest were designated non-typeable *S. aureus*; most of these strains were reported to possess a unique surface polysaccharide antigen, which contained polyribitol-phosphate-N-acetylglucosamine, a component of cell wall teichoic acid.

Knowledge of the type of CPs of the *S. aureus* isolates circulating on farms is essential in the formulation of the CP-based conjugate vaccine formulations because of their better immunogenicity due to T cell-dependence than the capsular polysaccharide formulations which are T cell-independent. Since the prevalence of CPs may be different in different countries, conjugate vaccine formulations specific for each farm or a broad-spectrum vaccine representing all the capsular types, surface-associated immune evasion molecules, conjugated to the most significant homologous or heterologous colonisation protein antigen(s), may be need to be formulated.

Because of the distribution of the different capsular types of *S. aureus* around the world may differ, it is important to know their prevalence in Australia and India used in this study as model developing country with backyard farming practices, in order to rationally select suitable vaccine candidates as vaccine candidates. However, there are no reports on the prevalence of different CP types of *S. aureus* causing bovine mastitis either in Australia or in India. Hence the objective of this study was to determine the distribution of different capsular phenotypes among the Australian and the Indian *S. aureus* strains isolated from clinical bovine mastitis cases.

Material and Methods

*S. aureus isolates*
One hundred and fifty four (154) fully characterised *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). In India, 260 strains were isolated from clinical cases of mastitis in cows from different parts of the country (Karnataka, Andhra Pradesh, Goa, Uttar Pradesh and Gujarat) and identified as *S. aureus* using the standard biochemical tests. Reference 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). *S. aureus* ATCC-55804 designated as serotype 336 was purchased from ATCC, USA. These isolates were grown on Mueller Hinton (MH) agar and subcultured in nutrient broth supplemented with 1% glucose and stored on cryobeads (Blackaby Diagnostics) or as glycerol (15%) broth stocks at -80°C.

**Production of antisera for serological typing**

Six week-old specific pathogen-free Quackenbush Swiss line 5 mice obtained from the Animal Resources Centre, Perth, Western Australia were and used for production of CP and antigen 336-specific antisera. All animal experiments were carried out with the approval of Curtin University’s Animal Ethics Committee.

Preparation of the different anti-CP type 1, 2, 5, 8 and SP-336 antisera was carried out according to Fournier et al. (1984) \(^4\). Briefly, bacterial suspensions of *S. aureus* CP types 1, 2, 5, 8 and SP-336 grown for 18 h on MH agar plated at 37°C and killed with 3% formalinised PBS followed by washing with PBS (5X). Mice were immunized every week for 5 weeks with formalin-killed *S. aureus*. The first three doses containing the equivalent to
5x10⁷, 1x10⁸ and 5x10⁸ CFU in 0.2mL respectively, were administered by the intraperitoneal route. For the fourth and fifth doses, the bacterin was mixed with equal proportion with Imject Alum (Thermo Scientific) and 0.2 mL containing an equivalent of 1x10⁹ and 5x10⁹ CFU, respectively, and administered subcutaneously one week apart. Mice were blood samples for collection of sera. The CP-/SP-specific antisera were cross-absorbed with non-capsulated S. aureus strain LAC, USA 300, followed by cross-absorption with the heterologous CP types CP1, CP2, CP5, and CP8 depending upon the desired CP specificity. For SP-336 serum, the same method was followed, initially cross absorbed with non-capsulated LAC USA followed by cross absorption with CP 1, 2 5 and 8.

**CP serotyping of S. aureus isolates**

Slide agglutination test was performed to determine the serotype of the strains. Each strain was grown overnight on MH agar and a single colony was resuspended in a drop of 0.9% normal saline in a clean glass slide. A drop of serum was added to it and observed for agglutination in less than 20 sec. The strains, which showed no agglutination against any of the four sera, were further tested for agglutination using anti-SP336 serum. Strains found negative with all the five specific sera were considered as non-typeable.

**CP genotyping of S. aureus isolates**

Genomic DNA was extracted from S. aureus using a kit (Geneworks, SA, Aust or Real Biotech Corp., Taiwan) Detection of the presence of cap1, cap2, cap5 and cap8 loci in all the strains was done by Polymerase Chain Reaction (PCR). The primers used for typing cap1 are

5′-AGG TCT GCT AAT TAG TGC AA-3′ (forward) and 5′-GAA CCC AGT ACA GGT ATC ACC A-3′ (reverse) with an expected product size of 550 bp and for cap2 are 5′-AGC AAT CGG TTA TTG CCG GTG-3′ (forward) and 5′-ATG ACA GTA AGG CAT
CAA GGT CG-3’ (reverse) with an expected amplicon size (non-specific). The PCR cycling parameters for both cap1 and cap2 were: denaturation at 94°C for 5 min followed by 94°C for 30 sec, Tm at 57°C (cap1) or 60°C (cap2) for 30 sec, 72°C for 60 sec with 25-30 cycles and final extension at 72°C for 5 min.

The primers used for typing cap5 and cap8 were 5’- ATG ACG ATG AGG ATA GCG-3’ (forward) and 5’- CTC GGA TAA CAC CTG TTG C-3’ (reverse) for cap5 and 5’- ATG ACG ATG AGG ATA GCG-3’ (forward) and 5’- CAC CTA ACA TAA GGC AAG-3’ (reverse) for cap8, respectively. The expected band sizes were 881 bp and 1148 bp for cap5 and cap8, respectively. Thermal cycling conditions were denaturation at 95°C for 5 min, 95°C for 30 sec, Tm of 55°C (cap5) or 52°C (cap8) for 30 sec, 72°C for 5 min with 25-30 cycles and the final extension at 72°C for 5 min.

The PCR products were analysed by agarose gel (1.5%) electrophoresis, Midori Green staining and UV trans-illumination. The positive controls included strains M, Smith Diffuse, Newman and USA 400 for cap1, cap2, cap5 and cap8, respectively, and LAC, USA 300 was used as negative control.

Statistical analysis

Correlation coefficients, represented as Pearson $r$ values, between the serological with the genotyping method, for CP1, CP5, CP8 positive and non-typeable S. aureus strains, were determined using Microsoft Excel, Windows 10.

Results

Prevalence of capsular or antigen 336 types in bovine S. aureus isolates
Genotyping of 154 Australian \textit{S. aureus} isolates revealed that 41 (26.62\%) and 50 (32.47\%) strains were positive for \textit{cap}5 and \textit{cap}8 (Table 1). None of the isolates were positive for the \textit{cap}1 locus. The primers for the \textit{cap}2 locus exhibited cross-reactivity with all the other three CP types producing amplicons of 700-800 bp (data not shown). A total of 63 (40.91\%) \textit{S. aureus} strains, which carried none of the three loci (\textit{cap}5, \textit{cap}8 or \textit{cap}1), were declared as negative by PCR (Table 1).

Serotyping of 154 \textit{S. aureus} isolates using CP-specific sera (Table 1) confirmed the genotyping results; 36 (23.4\%) and 49 (31.8\%) of the isolates revealed the presence of CP5 and CP8, respectively, whereas none was positive for CP1. However, 14 (9.1\%) strains were positive for CP2. The strains that were not agglutinated by any of the CP-specific sera were subjected to slide agglutination using anti-SP336 antiserum. Nine (5.8\%) isolates were positive for SP-336 and the remaining 46 (29.9\%) were declared as non-typeable.

Among the 260 Indian isolates, 10 (3.9\%), 221 (85\%) and 21 (8.1\%) were positive for \textit{cap}1, \textit{cap}5 and \textit{cap}8 loci, whereas the 8 (3.08\%) negative for all the three loci were considered as non-capsulated in genotyping. Serotyping revealed that 2 (0.8\%), 122 (46.9\%) and 34 (13.1\%) isolates were positive for CP1, CP5 and CP8, respectively. None of the remaining isolates was positive for either CP2 or SP-336, indicating that 102 (39.2\%) isolates were non-typeable (Table 1).

The correlation coefficient (\textit{r}) between the serological and genotyping methods for detection of CP types 1, 5 and 8, and the non-typeable \textit{S. aureus} isolates, was determined to be 0.97 and 0.66 for Australian and Indian \textit{S. aureus} isolates, respectively.
Discussion and conclusions

Given the role of surface-associated polysaccharides in the virulence of *S. aureus*, epidemiological investigations on the distribution of capsular and surface polysaccharide types among *S. aureus* isolates is important for rational design of a vaccine formulation against infection with *S. aureus*. Studies on human *S. aureus* capsular types have reported that 75-80% of all the isolates produce either CP5 or CP8.\(^\text{38,43,44}\) However, the prevalence of the CP serotypes (CP5 and 8) among the *S. aureus* strains isolated from cow’s milk ranges from as low as 14% to as high as 95%.\(^\text{29,31,45,46}\) Others\(^\text{47}\) reported that CP typing was superior to bacteriophage typing whereas another study\(^\text{48}\) had reported the capsular typing was less sensitive than genome typing yielding 26 different *S. aureus* types. Another study analyzed the genetic diversity of *S. aureus* isolated from subclinical mastitis cases in cows and reported the presence of 16 types and 24 subtypes.\(^\text{46}\) However, no information on their correlation with the different capsular types was reported.

The present study revealed that 64.3% of Australian bovine mastitis-associated *S. aureus* strains expressed capsule, of which, CP8 was predominant (31.8%), followed closely by CP5 (23.4%). A total of 60.8% of the Indian isolates expressed capsule, of which CP5 type was dominant (46.9%). In addition, 5.8% (nine of 154) of the Australian isolates were positive for SP-336, whereas none of the Indian isolates were positive for this antigen. The fact that about 40% of the Indian isolates were non-typeable suggests the possible existence of more diverse *S. aureus* populations in India than in Australia where about 30% of the isolates were non-typeable. These differences could be due to a multitude of factors including breed, husbandry and therapeutic practices, human-animal interface and various other environmental factors that are different between the two countries. However, it is difficult to draw any conclusions on whether *S. aureus* is transmitted between humans and animals in India because of a wide
variation in the distribution of the capsular phenotypes ranging from 21% to 63% for CP5 and 37% to 64% for CP8\textsuperscript{47,49,50}. There are no publications on the distribution of CP types of bovine mastitis-associated \textit{S. aureus} isolates from Australia, although only one study has genotypically characterised the CP5 and CP8 of Indian isolates\textsuperscript{51}. That study revealed that 60% of the cattle isolates and 20% of the goat isolates carried the \textit{cap5} gene, and 20% and 30% of cattle and goat isolates, respectively, carried the \textit{cap8} gene.\textsuperscript{51} However, only 20 \textit{S. aureus} isolates were used in that study whereas 260 isolates from different parts of India were used in this study, projecting a more reliable distribution of CP types in India.

Very few studies have compared the performance of genotyping versus serology in the typing of \textit{S. aureus} isolates. One study compared the prevalence of CP5 and CP8 types of \textit{Staphylococcus aureus} among isolates from intramammary infections in Argentine dairy cattle and found 64% of the isolates as genotypes \textit{cap5} or \textit{cap8} and 50% as CP5 or 8 serologically\textsuperscript{52}. In contrast, one study with human isolates, there was 100% correlation between capsular genotypes and phenotypes for CP5 and CP8.\textsuperscript{53} In this study, five (3.3%) Australian bovine mastitis-associated strains carrying \textit{cap5} and one strain (0.7%) carrying \textit{cap8} were negative by agglutination. More strikingly, 99 (38.1%) \textit{cap5} positive and eight (3.1%) \textit{cap1} positive Indian \textit{S. aureus} strains did not express respective capsular phenotype when judged by serological typing. In contrast, 13 (5%) Indian strains that were positive for CP8 by serology were negative by genotyping. The discordance between genotyping and phenotyping may be attributable to non-expression of respective capsule-encoding genes, possibly due to mutations\textsuperscript{30} or due to the difference in culture conditions \textit{in vivo} and \textit{in vitro}.\textsuperscript{54,55}
It is thus clear from this study that any surface-associated polysaccharide antigens-based vaccine formulation should not only include CP5 and CP8 types but also other capsular types, with or without antigen 336 or poly-N-acetyl glucosamine (PNAG), reported to be present in all *S. aureus* isolates\(^{56}\) for prevention of clinical bovine mastitis caused by *S. aureus*. The prevalence of 29.87% and 39.23% of non-typeable *S. aureus* strains in Australia and India, respectively, also highlights the need to explore the existence of other surface-associated polysaccharides including additional capsular phenotypes as proposed originally.\(^{43}\)

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**Conflict of interest**

The authors declare that they have no conflict of interests.
References


### Table 1

**Prevalence of capsular/surface polysaccharide phenotypes versus genotypes of *S. aureus* isolates from bovine mastitis cases in Australia and India**

<table>
<thead>
<tr>
<th>Country</th>
<th>Total no of <em>S. aureus</em> isolates</th>
<th>Detection of capsular type by slide agglutination test (SAT(^a))</th>
<th>NT(^b)</th>
<th>Detection of capsular type by PCR</th>
<th>Typing by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CP1</td>
<td>CP2</td>
<td>CP5</td>
<td>CP8</td>
</tr>
<tr>
<td>Australia</td>
<td>154</td>
<td>0</td>
<td>14</td>
<td>36</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(9.1)</td>
<td>(23.4)</td>
<td>(31.8)</td>
<td>(5.8)</td>
</tr>
<tr>
<td>India</td>
<td>260</td>
<td>2</td>
<td>0</td>
<td>122</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>(0.8)</td>
<td>(46.9)</td>
<td>(13.1)</td>
<td></td>
<td>(39.2)</td>
</tr>
</tbody>
</table>

\(^a\) NT = Non-typeable, SAT = Slide agglutination test; \(^b\) PDW = Primers did not work; \(^c\) PNA = Primers not available

Correlation coefficient (\(r\)) between SAT and PCR method for detection of CP1, CP5, CP8 and non-typeable *S. aureus* strains are 0.97 (Australia) and 0.66 (India).