This is the peer reviewed version of the following article:

Gogoi Tiwari, J. and Babra Waryah, C. and Sunagar, R. and Veeresh, H. and Nuthanalakshmi, V. and Preethirani, P. and Sharada, R. et al. 2015. Typing of Staphylococcus aureus isolated from bovine mastitis cases in Australia and India. Australian Veterinary Journal. 93 (8): pp. 278-282.,

which has been published in final form at http://doi.org/10.1111/avj.12349

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1 **Typing of *Staphylococcus aureus* isolated from bovine mastitis cases in Australia and**
2 **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 *cap*1, *cap*5 and *cap*8 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

54 Strategic Research Fund; MH, Mueller Hinton; ATCC, American type culture collection;
55 CFU, Colony forming unit; DNA, Deoxyribonucleic acid; UV, Ultra violet; PNAG, Poly-N-
56 acetyl glucosamine; CHIRI, Curtin Health Innovation Research Institute.

57

58 Introduction

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

72

73 Capsular polysaccharide (CP) is one of the most important virulence factors of *S. aureus*[24,25]
74 as it confers resistance to phagocytosis[26] and prolongs persistence of the pathogen in the
75 blood stream of the host.[27] Earlier serological studies suggested the existence of 11 different
76 serotypes of CP.[28,29] However, a more recent study[30] indicated that there were only four types
77 (CP 1, 2, 5 and 8), all the other reported types representing mutated versions. Most human
78 clinical isolates have been reported to express either CP5 or CP8, accounting for ~80% of the

79 total isolates from all sources.[31,26] The rest were designated non-typeable *S. aureus*; most of
80 these strains were reported to possess a unique surface polysaccharide antigen 336[29], which
81 contained polyribitol-phosphate-*N*-acetylglucosamine, a component of cell wall teichoic
82 acid.[26]

83

84 Knowledge of the type of CPs of the *S. sureus* isolates circulating on farms is essential in the
85 formulation of the CP-based conjugate vaccine formulations because of their better
86 immunogencity due to T cell-dependence than the capsular polysaccharide formulations
87 which are T cell-independent[32-34]. Since the prevalence of CPs may be different in different
88 countries[28,31,35-38], conjugate vaccine formulations specific for each farm or a broad-spectrum
89 vaccine representing all the capsular types, surface-associated immune evasion molecules[39],
90 conjugated to the most significant homologous or heterologous colonisation protein
91 antigen(s), may be need to be formulated.

92 Because of the distribution of the different capsular types of *S. aureus* around the world may
93 differ[35-38], it is important to know their prevalence in Australia and India used in this study as
94 model developing country with backyard farming practices, in order to rationally select
95 suitable vaccine candidates as vaccine candidates. However, there are no reports on the
96 prevalence of different CP types of *S. aureus* causing bovine mastitis either in Australia or in
97 India. Hence the objective of this study was to determine the distribution of different capsular
98 phenotypes among the Australian and the Indian *S. aureus* strains isolated from clinical
99 bovine mastitis cases.

100

101 **Material and Methods**

102 ***S. aureus isolates***

103 One hundred and fifty four (154) fully characterised *S. aureus* strains of Australian origin
104 isolated from clinical cases of mastitis in cows in Victoria and Queensland were generously
105 donated by Professor Margaret Deighton, (RMIT University), Dr. Sharon de Wet
106 (Queensland Biosecurity laboratory) and Dr. Justine Gibson (University of Queensland). In
107 India, 260 strains were isolated from clinical cases of mastitis in cows from different parts of
108 the country (Karnataka, Andhra Pradesh, Goa, Uttar Pradesh and Gujarat) and identified as
109 *S. aureus* using the standard biochemical tests.[40] Reference strains representing CP types 1
110 (strain M), 2 (strain Smith diffuse), 5 (strain Newman), 8 (USA 400 MW2) and a non-
111 capsulated strain (LAC, USA 300) were donated by Professor Gerald Pier (Harvard Medical
112 School, Boston, USA). *S. aureus* ATCC-55804 designated as serotype 336 was purchased
113 from ATCC, USA. These isolates were grown on Mueller Hinton (MH) agar and subcultured
114 in nutrient broth supplemented with 1% glucose and stored on cryobeads (Blackaby
115 Diagnostics) or as glycerol (15%) broth stocks at -80°C.

116

117 ***Production of antisera for serological typing***

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

122

123 Preparation of the different anti-CP type 1, 2, 5, 8 and SP-336 antisera was carried out
124 according to Fournier et al. (1984)[41]. Briefly, bacterial suspensions of *S. aureus* CP types-1,
125 2, 5, 8 and SP-336 grown for 18 h on MH agar plated at 37°C and killed with 3%
126 formalinised PBS followed by washing with PBS (5X). Mice were immunized every week
127 for 5 weeks with formalin-killed *S. aureus*. The first three doses containing the equivalent to

128 $5x10^7$, $1x10^8$ and $5x10^8$ CFU in 0.2mL respectively, were administered by the intraperitoneal
129 route.. For the fourth and fifth doses, the bacterin was mixed with equal proportion with
130 Imject Alum (Thermo Scientific) and 0.2 mL containing an equivalent of $1x10^9$ and $5x10^9$
131 CFU, respectively, and administered subcutaneously one week apart. Mice were blood
132 samples for collection of sera. The CP-/SP-specific antisera were cross-absorbed with non-
133 capsulated *S. aureus* strain LAC, USA 300, followed by cross-absorption with the
134 heterologous CP types CP1, CP2, CP5, and CP8 depending upon the desired CP specificity.
135 For SP-336 serum, the same method was followed, initially cross absorbed with non-
136 capsulated LAC USA followed by cross absorption with CP 1, 2 5 and 8.

137

138 ***CP serotyping of S. aureus isolates***

139 Slide agglutination test was performed to determine the serotype of the strains.[26] Each strain
140 was grown overnight on MH agar and a single colony was resuspended in a drop of 0.9%
141 normal saline in a clean glass slide. A drop of serum was added to it and observed for
142 agglutination in less than 20 sec. The strains, which showed no agglutination against any of
143 the four sera, were further tested for agglutination using anti-SP336 serum. Strains found
144 negative with all the five specific sera were considered as non-typeable.

145

146 ***CP genotyping of S. aureus isolates***

147 Genomic DNA was extracted from *S. aureus* using a kit (Geneworks, SA, Aust or Real
148 Biotech Corp., Taiwan) Detection of the presence of *cap*1, *cap*2, *cap*5 and *cap*8 loci in all the
149 strains was done by Polymerase Chain Reaction (PCR). The primers used for typing *cap*1 are
150 5'-AGG TCT GCT AAT TAG TGC AA-3' (forward) and 5'-GAA CCC AGT ACA GGT
151 ATC ACC A-3' (reverse) with an expected product size of 550 bp and for *cap*2 are 5'-AGC
152 AAT CTT CGG TTA TTG CCG GTG-3' (forward) and 5'-ATG ACG GTA AGG CAT

153 CAA GGT CG-3' (reverse) with an expected amplicon size (non-specific). The PCR cycling
154 parameters for both *cap*1 and *cap*2 were: denaturation at 94°C for 5 min followed by 94°C for
155 30 sec, Tm at 57°C (*cap*1) or 60°C (*cap*2) for 30 sec, 72°C for 60 sec with 25-30 cycles and
156 final extension at 72°C for 5 min.

157

158 The primers used for typing *cap*5 and *cap*8 were 5'- ATG ACG ATG AGG ATA GCG-3'
159 (forward) and 5'- CTC GGA TAA CAC CTG TTG C-3' (reverse) for *cap*5 and 5'- ATG
160 ACG ATG AGG ATA GCG-3' (forward) and 5'- CAC CTA ACA TAA GGC AAG-3'
161 (reverse) for *cap*8, respectively.[42] The expected band sizes were 881 bp and 1148 bp for *cap*5
162 and *cap*8, respectively. Thermal cycling conditions were denaturation at 95°C for 5 min,
163 95°C for 30 sec, Tm of 55°C (*cap*5) or 52°C (*cap*8) for 30 sec, 72°C for 5 min with 25-30
164 cycles and the final extension at 72°C for 5 min.

165

166 The PCR products were analysed by agarose gel (1.5%) electrophoresis, Midori Green
167 staining and UV trans-illumination. The positive controls included strains M, Smith Diffuse,
168 Newman and USA 400 for *cap*1, *cap*2, *cap*5 and *cap*8, respectively, and LAC, USA 300 was
169 used as negative control.

170

171 ***Statistical analysis***

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

175

176 **Results**

177 ***Prevalence of capsular or antigen 336 types in bovine S. aureus isolates***

178 Genotyping of 154 Australian *S. aureus* isolates revealed that 41 (26.62%) and 50 (32.47%)
179 strains were positive for *cap*5 and *cap*8 (Table1). None of the isolates were positive for the
180 *cap*1 locus. The primers for the *cap*2 locus exhibited cross-reactivity with all the other three
181 CP types producing amplicons of 700-800 bp (data not shown). A total of 63 (40.91%) *S.*
182 *aureus* strains, which carried none of the three loci (*cap*5, *cap*8 or *cap*1), were declared as
183 negative by PCR (Table1).

184

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

191

192 Among the 260 Indian isolates, 10 (3.9%), 221 (85%) and 21(8.1%) were positive for *cap*1,
193 *cap*5 and *cap*8 loci, whereas the 8 (3.08%) negative for all the three loci were considered as
194 non-capsulated in genotyping. Serotyping revealed that 2 (0.8%), 122 (46.9%) and 34
195 (13.1%) isolates were positive for CP1, CP5 and CP8, respectively. None of the remaining
196 isolates was positive for either CP2 or SP-336, indicating that 102 (39.2%) isolates were non-
197 typeable (Table 1).

198

199 The correlation coefficient (r) between the serological and genotyping methods for detection
200 of CP types 1, 5 and 8, and the non-typeable *S. aureus* isolates, was determined to be 0.97
201 and 0.66 for Australian and Indian *S. aureus* isolates, respectively

202

203 **Discussion and conclusions**

204 Given the role of surface-associated polysaccharides in the virulence of *S. aureus*,
205 epidemiological investigations on the distribution of capsular and surface polysaccharide
206 types among *S. aureus* isolates is important for rational design of a vaccine formulation
207 against infection with *S. aureus*. Studies on human *S. aureus* capsular types have reported
208 that 75-80% of all the isolates produce either CP5 or CP8.[38,43,44] However, the prevalence of
209 the CP serotypes (CP5 and 8) among the *S. aureus* strains isolated from cow's milk ranges
210 from as low as 14% to as high as 95%.[29,31,45,46] Others[47] reported that CP typing was superior
211 to bacteriophage typing whereas another study[48] had reported the capsular typing was less
212 sensitive than genome typing yielding 26 different *S. aureus* types. Another study analyzed
213 the genetic diversity of *S. aureus* isolated from subclinical mastitis cases in cows and
214 reported the presence of 16 types and 24 subtypes[46]. However, no information on their
215 correlation with the different capsular types was reported.
216
217 The present study revealed that 64.3% of Australian bovine mastitis-associated *S. aureus*
218 strains expressed capsule, of which, CP8 was predominant (31.8%), followed closely by CP5
219 (23.4%). A total of 60.8% of the Indian isolates expressed capsule, of which CP5 type was
220 dominant (46.9%). In addition, 5.8% (nine of 154) of the Australian isolates were positive for
221 SP-336, whereas none of the Indian isolates were positive for this antigen. The fact that about
222 40% of the Indian isolates were non-typeable suggests the possible existence of more diverse
223 *S. aureus* populations in India than in Australia where about 30% of the isolates were non-
224 typeable. These differences could be due to a multitude of factors including breed, husbandry
225 and therapeutic practices, human-animal interface and various other environmental factors
226 that are different between the two countries. However, it is difficult to draw any conclusions
227 on whether *S. aureus* is transmitted between humans and animals in India because of a wide

228 variation in the distribution of the capsular phenotypes ranging from 21% to 63% for CP5
229 and 37% to 64% for CP8[47,49,50]. There are no publications on the distribution of CP types of
230 bovine mastitis-associated *S. aureus* isolates from Australia, although only one study has
231 genotypically characterised the CP5 and CP8 of Indian isolates[51]. That study revealed that
232 60% of the cattle isolates and 20% of the goat isolates carried the *cap*5 gene, and 20% and
233 30% of cattle and goat isolates, respectively, carried the *cap*8 gene.[51] However, only 20 *S.*
234 *aureus* isolates were used in that study whereas 260 isolates from different parts of India
235 were used in this study, projecting a more reliable distribution of CP types in India.

236

237 Very few studies have compared the performance of genotyping versus serology in the typing
238 of *S. aureus* isolates. One study compared the prevalence of CP5 and CP8 types of
239 *Staphylococcus aureus* among isolates from intramammary infections in Argentine dairy
240 cattle and found 64% of the isolates as genotypes *cap*5 or *cap*8 and 50% as CP5 or 8
241 serologically[52]. In contrast, one study with human isolates, there was 100% correlation
242 between capsular genotypes and phenotypes for CP5 and CP8.[53] In this study, five (3.3%)
243 Australian bovine mastitis-associated strains carrying *cap*5 and one strain (0.7%) carrying
244 *cap*8 were negative by agglutination. More strikingly, 99 (38.1%) *cap*5 positive and eight
245 (3.1%) *cap*1 positive Indian *S. aureus* strains did not express respective capsular phenotype
246 when judged by serological typing. In contrast, 13 (5%) Indian strains that were positive for
247 CP8 by serology were negative by genotyping. The discordance between genotyping and
248 phenotyping may be attributable to non-expression of respective capsule-encoding genes,
249 possibly due to mutations[30] or due to the difference in culture conditions *in vivo* and *in*
250 *vitro*.[54,55]

251

252 It is thus clear from this study that any surface-associated polysaccharide antigens-based
253 vaccine formulation should not only include CP5 and CP8 types but also other capsular types,
254 with or without antigen 336 or poly-N-acetyl glucosamine (PNAG), reported to be present in
255 all *S. aureus* isolates[56] for prevention of clinical bovine mastitis caused by *S. aureus*. The
256 prevalence of 29.87% and 39.23% of non-typeable *S. aureus* strains in Australia and India,
257 respectively, also highlights the need to explore the existence of other surface-associated
258 polysaccharides including additional capsular phenotypes as proposed originally.[43]
259

260 **Acknowledgements**

261 The work was supported by grants through the Australia India Strategic Research Fund
262 [BF040038] from the Department of Innovation, Industry, Science and Research,
263 Commonwealth Government of Australia (to TKM), and the India-Australia Biotechnology
264 Fund [BT/Indo-Aus./04/06/2009] from the Department of Biotechnology, Ministry of
265 Science and Technology, Government of India (to NRH and SI). Thanks are also due to
266 Curtin University for providing the International Postgraduate Research Scholarship and the
267 Australian Postgraduate Award to JG-T in support her doctoral studies. The authors
268 acknowledge the provision of research facilities and the scientific and technical assistance of
269 the staff of CHIRI Biosciences Research Precinct Core Facility, Curtin University. The
270 authors are also grateful to Gerard Pier, Harvard University, for providing isolates expressing
271 specific capsular polysaccharides.
272

273 **Conflict of interest**

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

275

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Table 1

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

Country	No (%) of *S aureus* isolates												
	Total no of isolates	**Detection of capsular type by slide agglutination test (SAT[b])**					**NT[a] by SAT**	**Detection of capsular type by PCR**				**Typing by PCR**	
		CP1	**CP2**	**CP5**	**CP8**	**SP-336**		**CP1**	**CP2**	**CP5**	**CP8**	**SP-336**	**NT[a]**
Australia	154	0 (0)	14 (9.1)	36 (23.4)	49 (31.8)	9 (5.8)	46 (29.9)	0	PDW[c]	41 (26.6)	50 (32.5)	PNA[d]	63 (40.9)
India	260	2 (0.8)	0	122 (46.9)	34 (13.1)	0	102 (39.2)	10 (3.9)	PDW[c]	221 (85)	21 (8.1)	PNA[d]	8 (3.1)

[a]NT= Non-typeable, SAT[b] = Slide agglutination test; [c]PDW= Primers did not work, [d]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).