

The persistence of biofilm-associated antibiotic resistance of *Staphylococcus aureus* isolated from clinical bovine mastitis cases in Australia

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Abstract The aim of this investigation was to determine the persistence of biofilm-associated antibiotic resistance developed by methicillin-sensitive *Staphylococcus aureus* (MSSA),

of different capsular types, during biofilm formation. Because of superiority of the tissue culture plate (TCP) over the Congo Red Agar (CRA) method for measuring biofilm formation, it was used to determine the persistence of the antibiotic resistance developed by the isolates in biofilms. The antibiotic resistance was found to persist for 3–4 wk post-propagation as planktonic subcultures. Interestingly, some strains even developed resistance to vancomycin and/or teicoplanin. However, no association of either biofilm formation or persistent antibiotic resistance with the major capsular phenotype was observed. These observations highlight the potential significance of (a) determining the antibiograms of *S. aureus* subcultured from biofilms developed in vitro using the TCP method as well as from planktonic cultures for formulation of an optimal therapeutic strategy, and (b) continuing to identify predominant non-capsular antigens contributing to biofilm formation, regardless of the capsular phenotype for the development of an effective potentially broad-spectrum vaccine for prevention of bovine mastitis caused by *S. aureus*.

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Abbreviations

BAP	Biofilm-associated protein
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
TCP	Tissue Culture Plate
CRA	Congo Red Agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
PIA	Polysaccharide intercellular adhesin
PNAG	Poly-N-acetyl glucosamine
CP	Capsular polysaccharide

Introduction

One of the most common etiological agents of contagious mastitis (40.5 %) in cows is *Staphylococcus aureus* (Workineh et al. 2002). This presents a serious problem for the Australian dairy industry, the third largest rural industry, valued at \$12 billion at wholesale and \$2.9 billion for export, representing 9 % of world dairy trade (Dairy Australia 2011). Each case of mastitis is estimated to cost approximately \$200 per head with an average of three clinical mastitis cases per 100 cows on farm, mainly caused by this pathogen.

The major difficulty encountered in treating and managing staphylococcal mastitis has been the versatility of *S. aureus* in developing antimicrobial resistance, also noted with coagulase-negative *Staphylococcus* species (Melchior et al. 2007). In chronic and localised infections like mastitis, this may be largely dependent on the bacterium's ability to form biofilms (Mah and O'Toole 2001) in which bacterial cells can evade the antibiotic and host defences employed to inhibit colonization and subsequent infections. Biofilm formation starts with interaction of surface anchored bacterial factors, particularly the MSCRAMMs such as autolysin (Pozzi et al. 2012), an extracellular adhesion protein, PNAG (Maira-Laitren et al. 2004), eDNA (Mann et al. 2009), protein SasG (Geoghegan et al. 2010), and other recently described factors such as fibronectin binding proteins and clumping factor (Szczuka et al. 2012). While the role played by PNAG, also alternatively referred to as PIA, in infections caused by *S. aureus* has been well documented (Maira-Laitren et al. 2004) and reviewed extensively elsewhere (Archer et al. 2011; Arciola et al. 2012), there are no reports, to the best of our knowledge, on either the development of persistence of biofilm-associated antibiotic resistance of *S. aureus* or the emergence of resistance to different antibiotics.

On the other hand, PNAG-negative strains forming biofilms in *in vitro* settings have been reported (Beenken et al. 2004) highlighting the importance of other MSCRAMMs such as Bap, predominantly associated with isolates from chronic bovine mastitis infections (Tormo et al. 2005). Whether this is the case for infections *in vivo* is not known, as most clinical isolates of *S. aureus* produce PNAG (Szczuka et al. 2012) demonstrated to participate in biofilm formation (Kropec et al. 2005). Given the identification of MSCRAMMs other than PNAG as contributors to biofilm formation as highlighted above, it is important to continue the identification of the most critical biofilm-forming antigens of *S. aureus*, and other staphylococcal species, because it may assist in the development of an effective broad-spectrum vaccine or other antimicrobial therapies for infections caused by this versatile pathogen.

Majority of human *S. aureus* clinical isolates are reported to express either CP5 or CP8, representing 70–80 % of all the isolates (Nemeth and Lee 1995; Fattom et al. 1996),

although their percentage distribution in bovine mastitis has been reported to be variable (Han et al. 2000), with non-typeable strains expressing a different surface antigen 336, a cell wall ribitol teichoic acid substituted with an *N*-acetyl glucosamine monosaccharide (Verdier et al. 2007). Non-typeable *S. aureus* isolates do not have a functional genetic locus for either CP5 or CP8 synthesis (Cocchiario et al. 2006). However, the potential role of capsules of *S. aureus* in the colonisation process, the first step in biofilm formation, has not been investigated. This perhaps is due to acceptance of the “golden rule” that the major function of capsule is protection of the pathogen from opsonophagocytosis (Nemeth and Lee 1995; Gordon and Franklin 2008). Interestingly, the capsules of *S. pneumoniae*, a different gram-positive coccus, were reported to enhance pneumococcal colonisation by limiting mucus-mediated clearance (Magee and Yother 2001; Nelson et al. 2007), hence our interest in determining the potential association of the major capsular phenotypes of *S. aureus* with biofilm formation, and as a corollary, antibiotic resistance.

Therefore the major aims of this study were to (1) investigate the biofilm-forming potential and the impact on the emergence of persistent antibiotic resistance developed by methicillin-sensitive *S. aureus* (MSSA) clinical isolates in biofilms and (2) determine potential association of biofilm formation with the major capsular phenotypes.

Material and methods

Bacterial strains and culture conditions

One hundred and sixty (160) phenotypically characterised, *S. aureus* isolates from bovine mastitis cases in Victoria and Queensland were cultivated in nutrient broth supplemented with 1 % glucose. The accredited control *S. aureus* strains used in this investigation were strain M (CP1), Smith diffuse strain (CP2), USA 400 MW2 (CP8), Strain Newman and USA 100 NRS 648 (CP5), CP-negative isolates (USA 300 LAC and USA 300 NRS 648) and a strong biofilm-forming strain ATCC 29213 as a positive control.

Screening of *S. aureus* isolates for methicillin-resistance

Chromogenic MRSA ID plates (Biomerieux 2011) were used to check for bacterial growth in the presence of cefoxitin and amplification of the *mecA* gene product using polymerase chain reaction as reported elsewhere (Murakami et al. 1991).

Detection of biofilm production

Congo Red Agar method Colony phenotypes on CRA were determined as described by Freeman et al. (1989), with the

modification of incubating of the streaked plates for 96 h to ensure maximal biofilm formation (data not shown).

Tissue Culture Plate method Aliquots (200 µL) of nutrient broth, supplemented with 1 % glucose, were inoculated with *S. aureus* strains in 96-well sterile microtitre plates and incubated overnight at 37 °C to ensure adequate biofilm production. The two methods used to assess biofilm formation were (1) sodium metaperiodate treatment method (Kogan et al. 2006) and (2) crystal violet staining method (Christensen et al. 1985). The arbitrary cut-off point used for nonbiofilm-producing strains was 0.120 (Stepanovic et al. 2000). The arbitrary cut-off points used for weakly, intermediate or strongly adherent bacterial populations were 0.130–4.00×0.120 $A_{570\text{nm}}$, 4.10–5.90×0.120 $A_{570\text{nm}}$ and greater than 6×0.120 $A_{570\text{nm}}$, respectively.

Capsular typing DNA extracts of 160 strains were run against Cap5 and Cap8 primers (Moore and Lindsay 2001). Primers for Cap5 forward were 5'-ATGACGATGAGGATAGCG-3' and reverse 5'-CTCGGATAACACCTGTTGC-3' with a T_m of 60 °C. Primers for Cap8 forward were 5'-ATGACGATGAGGATAGCG-3' and reverse 5'-CACCTAACATAAGGCAAG-3' with a T_m of 53 °C. Expected band size for Cap5 and Cap8 were 881 and 1,148 bp, respectively. The PCR products were separated on a 1.5 % agarose gel in 1× TAE buffer, stained with SYBR® Safe DNA Gel Stain (Invitrogen) and visualized using a UV trans-illuminator.

Amplification of the *mecA* gene Detection of the *mecA* gene (Murakami et al. 1991) was carried out using the forward primer 5'-AAAATCGATGGTAAAGGTTGGC-3' and reverse primer 5'-AGTTCTGCAGTACCGGATTTGC-3' with a T_m of 52 °C. The PCR products were separated on 1.5 % agarose gel with 1× TAE buffer, stained with SYBR® Safe DNA Gel Stain (Invitrogen) and visualised the expected band size of 533 bp using a UV trans-illuminator.

Antibiotic susceptibility tests Antibiotic susceptibility tests were performed using the CDS method (Bell et al. 2009) on biofilm-producing *S. aureus* isolates. The antibiotic sensitivities of bacteria subcultured from the biofilms were compared with the planktonic/free-floating bacteria using Sensitest plates (PathWest). The antibiotics used for susceptibility were benzylpenicillin (*P* 0.5), 0.5 µg per disc, cefoxitin (FOX 10), 10 µg per disc, cephalixin (CL 100), 100 µg per disc, ciprofloxacin (CIP 2.5), 2.5 µg per disc, cotrimoxazole (SXT 25), 25 µg per disc, erythromycin (E 5), 5 µg, linezolid (LZD 10), 10 µg, mupirocin (MUP 200), 200 µg, rifampicin (RD 1), 1 µg, teicoplanin (TEC 15), 15 µg/disc, tetracycline (TE 10), 10 µg per disc, and vancomycin (VAN 5), 5 µg per disc (Oxoid). Zones of

inhibition of 6 mm or greater in diameter were interpreted as susceptible. Exceptions to this rule were vancomycin (VA5) and teicoplanin (TEC 15), for which a 2-mm diameter was used as the cut-off point.

Results

Using the CRA method, only 54 of the 160 isolates (33.8 %) were positive for biofilm formation. However, all the 160 MSSA isolates were positive for biofilm formation, graded as weak, moderate or intermediate and strong, by the TCP method (Table 1). This demonstrated the superiority of the TCP over CRA method for detection of biofilm formation. It was also observed that there was no association of the capsular phenotype of *S. aureus* and biofilm formation because all the encapsulated as well as the even the non-typeable strains that do not express the capsular antigens (58/160 strains by the TCP method; 14/160 strains by the CRA method) were found to produce biofilms (Table 1).

The accredited control *S. aureus* used in this investigation were found to range from nonbiofilm-producing (Strain M—CP1, Smith diffuse—CP2), weak biofilm producers (USA 300LAC—CP Neg and USA 100 NRS 64—CP5), moderate biofilm-producing (USA 300 NRS 680—CP Neg) to strong biofilm-producing strains (Strain Newman—CP5, USA 400 MW2—CP5 and the ATCC2913—CP8) (data not shown).

None of the 160 Australian bovine mastitis clinical isolates were found to be MRSA using the Chrom ID MRSA plates [Biomerieux] and absence of the *mecA* gene product by specific PCR. However, out of the accredited CP types, USA NRS 648 (CP5), USA 400 MW2 (CP8), USA 300 NRS 680 (CP Neg) and USA 300 LAC (CP Neg) were confirmed to be MRSA. Of the 160 Australian MSSA isolates, 63 % were

Table 1 Comparison of CRA versus TCP methods for assessment of biofilm formation by methicillin-sensitive (MSSA) isolated from bovine mastitis cases

Capsular phenotype of MSSA isolates	Method used for assessment of biofilm formation	
	TCP ^a	CRA ^b
CP5	44/160 (27.5 %) ^c	9/160 (5.6 %) ^c
CP8	58/160 (36.3 %) ^c	31/160 (19.4 %) ^c
NT ^d	58/160 (36.3 %) ^c	14/160 (8.8 %) ^c
Total	160/160 (100 %) ^c	58/160 (33.8 %) ^c

^a TCP denotes tissue culture plate method

^b CRA denotes Congo Red Agar method

^c Parenthesis denotes the calculated percentage of positive strains

^d NT denotes non-typeable

encapsulated (44/160—CP5 and 58/160—CP8 type), the remaining 36.25 % being non-typeable as CP5 or CP8.

Antibiotic susceptibility tests performed on 34 strong biofilm-forming *S. aureus* isolates, including CP type 8-USA 400 Strain MW2, revealed multiple antibiotic resistances when grown as biofilms, with two Australian isolates (BoAISRF S.aur40 and BoAISRF S.aur41) and the USA 400 Strain MW2 developing resistance to vancomycin. The isolate BoAISRF S.aur41 also developed resistance to teicoplanin (Table 2).

The fact that 12 % of the Australian clinical mastitis MSSA isolates developed resistance to more than one antibiotic, as did the USA 400 MW2 strain, including vancomycin and/or teicoplanin, represents a matter of serious concern.

In order to determine whether the developed antibiotic resistance was transient or persistent, *S. aureus* scooped from the biofilms of the above-mentioned 5 isolates stored at 4 °C and re-tested for susceptibility against the same antibiotic after subculturing at days 7, 14, 21 and 30. Antibiotic resistance was found to persist until 3–4 weeks, reverting back to susceptibility by week 4 post-propagation as planktonic subcultures.

Discussion

Comparison of the TCP with the CRA method for detection of biofilm formation, using four accredited CP types, three non-encapsulated strains and 160 MSSA isolates from clinical bovine mastitis cases in Australia, revealed that substantial number (66.25 %) of biofilm-producing isolates remained undetected when the CRA method was used to detect biofilm production. On the other hand, 100 % of the

MSSA isolates were found to produce biofilms using the TCP method. This conclusion is in contrast to previous reports by other investigators (Oliviera et al. 2006; Begum et al. 2007). Our decision to use the incubation period of 96 h is validated by the report by Begum et al. (2007) who, using fluorescent in situ hybridisation, reported that the biofilm-forming ability of *S. aureus* increased with an increase in the incubation time (34.6 % at 24 h, 69.2 % at 48 h and 80.8 % at 72 h post-incubation). Our conclusion is based on the assumption that the antigens precipitating biofilm formation by the CRA plate and TCP methods are the same, an area needing further investigations. However, regardless of the method adopted for detection of biofilm-producing *S. aureus*, there appeared to be no association of the capsular phenotype with biofilm formation since many non-encapsulated *S. aureus* strains were also found to form weak, moderate, or strong biofilms, as reported recently for *S. aureus* strains of human origin (Szczuka et al. 2012).

In addition to confirming a multitude of the previous reports on development of antibiotic resistance in biofilms (Christensen et al. 1985; Stepanovic et al. 2000; Mah and O'Toole 2001; Melchior et al. 2007; Kogan et al. 2006; Tormo et al. 2005; Oliveira et al. 2006; Begum et al. 2007), our investigation is the first one to demonstrate that antibiotic resistance, developed by MSSA strains during biofilm formation and became persistent as judged by retention of antibiotic resistance in planktonic subcultures until days 21 to 30. Subsequently, these strains reverted back to antibiotic sensitivity. The mechanism underpinning persistence of antibiotic resistance of *S. aureus* in biofilms was not investigated in this study but warrants further investigation(s).

Emergence of persistent antibiotic resistance by *S. aureus* is a matter of great concern in the therapy of staphylococcal

Table 2 A comparison of antibiotic sensitivity of planktonic versus biofilm-associated *S. aureus* isolates

Antibiotic (µg per disc)	Biofilm-associated <i>S. aureus</i>				
	BoAISRF S.aur32	BoAISRF S.aur40	BoAISRF S.aur41	BoAISRF S.aur8	USA 400 MW2
Tetracycline (10)	S→R	S→R	S→R	S	S→R
Teicoplanin (15)	S	S	S→R	S	S
Rifampicin (1)	S	S	S	S	S
Cefoxitin (10)	S	S	S	S	R
Penicillin (0.5)	S	S	S	S	R
Erythromycin (5)	S→R	S	S	S	S→R
Ciprofloxacin (2.5)	S	S	S→R	S	S
Co-trimoxazole (25)	S	S	S→R	S	S
Mupirocin (200)	S	S	S	S	S
Linezolid (10)	S	S	S	S	S→R
Cephalexin (100)	S	S	S	S	R
Vancomycin (5)	S	S→R	S→R	S	S→R

Arrows indicate change in resistance. BoAISRF denotes bovine *S. aureus*; S Susceptible; R Resistant; S→R denotes acquisition of resistance to the respective antibiotic used in testing antibiotic susceptibility

local and systemic infections, particularly given the change in the resistance phenotype, as demonstrated for select *S. aureus* strains in this investigation, to vancomycin or teicoplanin, the last resort antibiotics. It is hypothesised that selection of antibiotics for treatment of mastitis, based on the susceptibility of *S. aureus* in both the planktonic and the biofilm-embedded/associated states, may yield better therapeutic outcomes particularly in stubborn cases of bovine mastitis as recently suggested for human MRSA infections (Khan et al. 2011). Furthermore, the lack of association of the capsular phenotypes of *S. aureus* with biofilm formation ruled out a potential role of one of the three major polysaccharide MSCRAMMs in colonisation of the host by *S. aureus*, hence warranting comparative studies on the protective potential of capsular versus the non-capsular biofilm-forming antigen(s)-based vaccine(s).

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