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Prevalence of *Staphylococcus aureus* strains in an Australian cohort, 1989-2003: evidence for low prevalence of the toxic shock toxin gene

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Abstract:

Purpose

To determine the prevalence of the toxic shock toxin gene (*tst*) and to enumerate circulating strains of methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) in Australian isolates collected over two decades.

Methods

Isolates were assayed using real-time PCR for *mecA, nuc*, 16S rRNA, 8 single nucleotide polymorphisms (SNPs) and for five binary genes (*pvl*, *cna*, *sdrE*, pUB110, pT181). Two real-time PCR assays were developed for *tst*.

Results

The 90 MRSA isolates belonged to CC239 (39 in 1989, 38 in 1996 and 10 in 2003), CC1 (2 in 2003) and CC22 (1 in 2003). The majority of the 210 MSSA isolates belonged to CC1 (28), CC5 (24), CC78 (23), CC15 (22) and CC45 (22). Only 18 isolates were *tst*-positive and only 15 were *pvl*-positive. Nine MSSA isolates belonged to 5 binary types of ST93 including 2 *pvl*-positive types.

Conclusions

The proportion of *tst*-positive and *pvl*-positive isolates was low and no significant increase was demonstrated. Dominant MSSA clonal complexes were similar to those seen elsewhere exception for CC78. CC239 MRSA (AUS-2/3) was the predominant MRSA but decreased significantly in prevalence while CC22 (EMRSA-15) and CC1 (WA-1) emerged. Genetically diverse ST93 MSSA predated the emergence of ST93-MRSA (the Queensland clone).

Keywords:

Toxic shock syndrome, TSST-1, *Staphylococcus aureus*, MRSA, Australia

Introduction:

The pathogenicity of *Staphylococcus aureus* can in part be attributed to the production of pyrogenic toxins such as toxic shock toxin-1 (TSST-1) [1]. TSST-1 is a 21.9-kDa protein that is encoded by the *S. aureus* toxic shock toxin gene (*tst*) gene [2]. The release of TSST-1 into the bloodstream may give rise to a variety of severe clinical conditions, such as toxic shock syndrome (TSS), sudden infant death syndrome, and Kawasaki syndrome. The *tst* gene is present in up to 70% of the *S. aureus* strains isolated from patients with TSS. Without appropriate therapy, lethal shock may develop within 24h after the onset of symptoms. Although most cases of TSS are associated with tampon use, an increasing number of cases are related to localized infections, surgical complications and insect bites [1-4].

There has been a worldwide increase in the frequency of clusters of TSS since the 1980s, but this has not been noted specifically in cases reported from Australia. In 1983 the Communicable Diseases Intelligence reported 11 cases of toxic shock in 1981, 7 in 1982 and 4 in 1983 in Australia [5]. The Therapeutic Goods Administration stated that between 1982 and 1994 there were 38 reports of TSS in Australia, of which 3 cases were fatal and 10 were female non-menstrual cases [6]. In 1996 Robinson and Peel reported the result of 2 surveys to identify TSS cases 1990-1994. Their survey revealed 11 possible cases of TSS from intensive care units (ICUs) in Australia and New Zealand and 12 TSS cases from Victoria, Australia [7]. A search by Donovan *et al* for possible fatal cases of TSS, by reviewing records of females whose deaths were registered in 1978-1979, revealed that unrecognized TSS could not be excluded as the cause of one of the deaths [8]. A number of isolated case reports of TSS have been published including TSS associated with newly diagnosed type I diabetes mellitus [9] and TSS in a male complicated by oliguric acute renal failure [10]. Reports of shock associated with *S. aureus* infection which resembled TSS but not reported as such, include the death of 12 children after receiving a *S. aureus* contaminated diphtheria toxin-antitoxin mixture and a case of late-onset warfarin necrosis [11, 12]. In 2005 a case of menstrual TSS was reported by MacIsaac et al indicating that staphylococcus toxic shock syndrome is still a problem [13]. The paucity of ongoing reports on TSS and absence of reports indicating an increase of TSS in Australia raises the question of whether the TSST-1 gene is common in *S. aureus* in Australia. It has been postulated that modifications to the host population, such that the fitness of organisms already carrying TSST-1 genes increases, could result in an increase in TSS [14].

The aims of this study were to determine the prevalence of the *tst* gene and to enumerate the circulating strains of *S. aureus* including methicillin-resistant *S. aureus* (MRSA) in a substantial Australian collection of isolates encompassing 1989-2003.

Materials and methods:

Selection of archival isolates

300 *S. aureus* isolates collected at Princess Alexandra Hospital as part of Australian Group for Antimicrobial Resistance (AGAR) 1989, 1996 and 2003 national staphylococcal surveys were examined. The same sampling methodology was used in all three year groups and there was no distinction made between community- and healthcare-associated isolates. The isolates were from specimens submitted for clinical diagnosis and not for infection control screening and duplicates were excluded [15].

After the isolates were retrieved from storage at minus 80°C, there were 91 pure *S. aureus* isolates from 1989, 104 from 1996 and 105 from 2003.

DNA extraction

Isolates were grown on horse blood agar in air at 37°C overnight. 1 μL loop of pure 24 hour colonies were emulsified into 250 μL water in 1.5mL simple prelabelled microtube and DNA extraction was performed using the MagNA Pure LC Total Nucleic Acid Isolation Kit on the Roche MagNA Pure instrument.
Amplification of the 16S ribosomal DNA was used to confirm the quality of each DNA extract and the absence of PCR inhibitors.

Detection of tst gene

Two assays were developed for the detection of the *tst* gene, using the two primer sets previously described in the literature (Table 1). Primer sets were supplied by Sigma Genosys. The *tst* gene was detected using real-time PCR with SYBR green on the RotorGene 3000 (Corbett Research, Mortlake, Australia).
The conditions of the assay were:

50^{o}C 2 min, 95^{o}C 2 min, 40 cycles of 95^{o}C 15 sec, 56^{o}C 20 sec, 72^{o}C 35 sec; Melt 72^{o}C to 99^{o}C at 1^{o}C intervals.

The gene was considered detected if the cycle threshold (Ct) was crossed in a predetermined Ct interval within which all positive control strains were positive and all negative control strains undetected.

Strain typing

Real-time PCR assays for *mecA, nuc* and 16S rRNA genes were performed on all isolates to confirm the identity and methicillin resistance, based on publications by Barski et al and Unal et al.[16, 17].

Real-time PCR typing of *S. aureus* is based on previous publication by Huygens *et al*. [18], for single nucleotide polymorphisms (SNP) derived from the multi locus sequence typing (MLST) database of 7 housekeeping genes. The method involves 8 PCRs for *arcC*210, *tpi*241 + *tpi*243, *arcC*162, *gmk*318, *pta*294, *tpi*36 *(tpi*36*C and *tpi*36*T) and *pta*383. It is able to distinguish between major clonal complexes (CCs) and is largely concordant with the population structure of *S. aureus* as determined by MLST. A single SNP-based PCR to detect *aroE525G* was added to confirm the identity of ST93 (the Queensland clone) when the appropriate 7 SNP profile (TGGTTCTA)was obtained. The binary genes interrogated were *pvl*, *cna*, *sdrE*, pUB110 and pT181 according to Huygens *et al* [18]. The presence of the genes was detected using real-time PCR with SYBR green on the RotorGene 3000 (Corbett Research, Mortlake, Australia). The genes were considered detected if the threshold was crossed within the predetermined Ct.

Statistical analysis

Confidence interval calculations for proportions and chi-square analysis for probability estimates were performed using the Vassarstats Web Site (http://faculty.vassar.edu/lowry/VassarStats.html).

Results:

Prevalence of clonal complexes of MRSA and methicillin-sensitive S. aureus (MSSA)

SNP profiles of the isolates correlated with 21 CCs or sequence types (STs): 91 with CC239, 28 with CC1, 24 with CC5, 23 with CC78, 22 with CC15 and 22 with CC45. CC239 was the major strain for 1989 (43/91) followed by CC45 (10/91) and CC15 (6/91). CC239 was again the major strain in 1996 (38/104), followed by CC1 (13/104)

and CC78 (11/104). In 2003, CC5 was predominant with 15/105, followed by both CC1 and CC15 with 11/105, and both CC239 and CC78 with 10/105 (Table 2).

90 isolates were confirmed as MRSA and SNP profiles of the isolates correlated with 3 CCs: 87 with CC239, 2 with CC1 (WA-1) and 1 CC22 (EMRSA-15). There were 39 (42.9%; 95% confidence interval (CI) 33.2%-53.1%) MRSA isolates in the 1989 collection and 38 (36.5; 95% CI 27.9%-46.1%) in 1996, all CC239 (AUS-2/3). In 2003 there were only 13 (12.4%; 95% CI 7.4%-20.0%) MRSA isolates (P <0.0001) of which 10 were CC239, 2 CC1 (WA-1) and 1 CC22 (EMRSA-15).

Of 210 MSSA isolates 26 (12.4%) were CC1, 24 (11.4%) CC5, 23 (11.0%) CC78, 22 (10.5%) CC15, 22 (10.5%) CC45 and 17 (8.1%) CC30. The predominant strains for 1989 were CC45 (10/52) and CC15 (6/52), for 1996 were CC1 (13/66) and CC78 (11/66) and for 2003 were CC5 (15/92), CC15 (11/92) followed by CC78 (10/92). The yearly variation in prevalence of major clones was not statistically significant. Nine MSSA isolates belonged to ST93: 4 were in 1989, 2 in 1996 and 3 in 2003. Five binary types of ST93 were described including two in which *pvl* was present. Three of the five types including both *pvl*-positive types were seen in the isolates from 1989. Overall five MSSA isolates were *pvl*-positive in each year. No MRSA were *pvl*-positive.

Prevalence of tst positive isolates

The *tst* gene was present in 18 isolates (6%; 95% CI 3.8%-9.3%) overall. The annual prevalence did not vary significantly (P = 0.289) with 3 (3.3%; 95% CI1.1%-9.3%) isolates positive in 1989, 6 (5.8%; 95% CI 2.7%-12.0%) positive in 1996 and 9 (8.6%; 95% CI 4.6%-15.5%) in 2003. Based on the SNP type correlation, CC30 was predominant with 11 isolates, followed by 2 isolates correlating with CC45 and 2 with CC8, followed by the rest (Table 3).

CC30 was the predominant strain for *tst* positive isolates in all three years, with 2/3 isolates *tst* positive in 1989, the remaining *tst* positive isolate for 1989 was the sole isolate with a SNP profile consistent with ST67, ST198, ST395, ST426, ST479, ST66, ST68, or ST520. In 1996 4/6 CC30, 1/7 CC45 and 1/1 CC72 isolates were *tst*-positive and in 2003 5/9 CC30, 2/6 CC8, 1/5 CC45 and 1/3 ST93 isolates were *tst* positive.

tst positive isolates were from a variety of sites, none were from the female genital tract and none of the patients were considered to have TSS. There were equal numbers of male and female patients with isolates positive for the *tst* gene. Ages of patients ranged from 14 to 86 years. None of the *tst* positive isolates had the *mecA* or *pvl* gene detected. The binary gene results are summarised in Table 3.

Discussion:

TSST-1 is a superantigenic toxin encoded by the *tst* gene in *S. aureus* and is associated with TSS amongst various clinical diseases. Methods based on detection of the toxin depend on the concentration of toxin expressed and can be negatively influenced by various factors [19]. Detection of the *tst* gene by PCR can overcome these difficulties if a representative clinical isolate is available. In this study we have shown that the prevalence of *tst* in a substantial collection of *S. aureus* is low and has not changed significantly over two decades. This provides a plausible explanation for the low reportage of staphylococcal TSS in Australia.

The genotyping data in this study provides a unique picture of prevalent *S. aureus* strains in Australia over two decades as previous studies have been restricted to MRSA or to a short time-frame [20, 21]. Detection of the binary genes and integrated plasmids adds further discrimination and potentially significant information on the prevalence of toxins of clinical interest such as *tst* and *pvl*.

Among the 90 MRSA isolates CC239 MRSA (AUS-2/3) was the overall predominant strain and also the sole strain in 1989 and 1996. CC1 MRSA (WA-1) and CC22 MRSA (EMRSA-15) only appeared in 2003. However, the total number of MRSA isolates decreased significantly in that year due to a marked decrease in CC239. The presence of CC239 (which has previously been shown to be synonymous with ST239-MRSA-III) as the only MRSA clone in the 1989 and 1996 surveys is in keeping with previous studies. ST239-MRSA-III (also known in Australia as EA-MRSA or AUS-2/3 MRSA) has been the dominant healthcare-associated strain of MRSA in Australia since the late 1970s. However, to our knowledge molecular typing of MRSA in Queensland by a method referrable to MLST had not been undertaken on isolations made prior to 2000. Our results provide further evidence of the ubiquity of this international epidemic clone in eastern Australia in the late 20th century. The importation of other strains of MRSA such as EMRSA-15 into Australia and the

emergence of still more local strains, including WA-1, since 2000 has been described previously and is in keeping with our findings [15, 20]. Of 210 MSSA isolates, the major clones present were CC1, CC5, CC78, CC15, CC45 and CC30. Of these all but CC5 and CC78 were major clones present in the Oxfordshire collection [22]. However, the CC5 was also represented by a substantial number of isolates in that study but was characterised as a minor group at that time. CC78, on the other hand, was not present at all. Australia has been the major source of STs (6 of 18) belonging to this CC in the MLST database. Most registered isolates (22 of 28) have been MRSA [23].

MSSA isolates belonging to ST 93 were detected in each survey year. This sequence type is a singleton apparently unique to Australia and distinct from other lineages [23, 24]. The MRSA clone of this sequence type was first described in Queensland in 2000 [25]. A subsequent clinical cohort study conducted in the same region in 2004 and 2005 identified five binary types among MSSA isolates and only one for ST93-MRSA (a *pvl*-positive clone) [21]. Similarly, five binary types of ST93-MSSA were detected in this study. Three of these five types, including two *pvl*-positive types, were present among the 1989 isolates thus demonstrating that a diverse population of ST93 was present in south-east Queensland prior to the emergence of the Queensland clone by acquisition of SCC*mec* type IV.

TSST-1 has been frequently associated with MSSA ST30, but recently community-acquired and hospital acquired *tst*-positive MRSA belonging to ST5 and ST30 has been reported in Europe and Japan [26-30]. All of our *tst*-positive isolates were MSSA and the perhaps predictably the majority of these (11/18) belonged to CC30. In addition, the majority (11/17) of all CC30 isolates were *tst* positive.

The number of *pvl*-positive isolates was surprisingly low: 15 MSSA overall (5%) with 5 in each year group. A clinical cohort study in 2004-5 in the same region showed a pvl prevalence of 16% in 192 MSSA isolates [21]. In addition, the majority (55%) of non-multiresistant MRSA isolates in the same study were also *pvl*-positive thus suggesting that there has been an increase in the prevalence of *pvl* in circulating *S. aureus*. In the current study most *pvl*-positive isolates belonged to CC30 (5) or ST93

(5), the same lineages as the major *pvl*-positive community-associated MRSA clones that have expanded over the last decade.

The *tst*-positive isolates in this study were from a variety of sites including superficial sites and deep sterile sites in patients of widely varying age. While none of these patients had TSS this is not surprising as production and penetration of sufficient concentration of TSST-1 is required for development of the toxic shock syndrome [31] and only occurs in a subset of infections due to *tst*-positive strains.

There have been recent overseas publications drawing attention to infection due to TSST-1 positive *S. aureus* [26, 27, 32, 33]. For example, TSST-1 has been involved in an outbreak of neonatal toxic shock-like exanthematous disease in a neonatal intensive care unit in Japan [32]. The emergence of TSST-1 positive community-associated MRSA strains may lead higher rates of TSS among the young with attendant impact on infection control and empiric therapeutic strategies [26]. The recently reported *tst*-positive MRSA clones ST5 and ST30 have been associated with both hospital-acquired and community-acquired infections causing a variety of clinical syndromes, including TSS and suppurative infections. Recent overseas publications have reported that *tst*-positive MSSA strains belonging to ST1, ST8, and ST30, are a potential source of *tst*-positive CA-MRSA and that the pulsotype of *tst*-positive MRSA clones have been found to differ from that of MSSA by a single band involving the SCC*mec* element. These findings suggest that the *tst*-positive MRSA clones may have emerged from their respective MSSA counterparts and has the same sequence type (ST5) of two pandemic nosocomial MRSA clones [27, 34]. These publications support the desirability for ongoing surveillance of MRSA and MSSA harbouring the *tst* gene as an aid to targeted infection control.

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Tables:

Table 1: Primer sets for *tst* assays [1, 35]

Assay	Primer	Sequence	Location
1	Forward	TCATCAGCTAACTCAAATACATGGATT	537-563
	Reverse	TGTGGATCCGTCATTCATTGTT	603-624
2	Forward	CCCTTTGTTGCTTGCGACA	38-57
	Reverse	CCCACTACTATACCAGTCTAGCAAATCC	141-170

Table 2: SNP profiles and corresponding CC/ST for *S. aureus* isolates from 1989, 1996 and 2003.

MLST correlation	Profile	1989 n (%)	1996 n (%)	2003 n (%)	Total n (%)
CC1	CGATAACT/ TGATTACT	4 (4.4)	13 (12.5)	11 (10.5)	28 (9.3)
CC15	CGATAACA	6 (6.6)	5 (4.8)	11 (10.5)	22 (7.3)
CC20	CGATTACT		2 (1.9)	2 (1.9)	4 (1.3)
CC22	CGGTTACA		1 (1)	2 (1.9)	3 (1)
CC239	TGAAACCA	43 (47.3)	38 (36.5)	10 (9.5)	91 (30.3)
CC25	CGGTAACA		2 (1.9)	2 (1.9)	4 (1.3)
CC30	TGGATCCA	3 (3.3)	7 (6.7)	7 (6.7)	17 (5.7)
CC45	CGGATCCA	10 (11)	7 (6.7)	5 (4.8)	22 (7.3)
CC5	CGATTACA	2 (2.2)	7 (6.7)	15 (14.3)	24 (8)
CC509	CGGTTCCA	3 (3.3)	3 (2.9)	4 (3.8)	10 (3.3)
CC59, 121, 133	TGGTTCTA	2 (2.2)		1 (1)	3 (1)
CC72	CGATTCCA	1 (1.1)	1 (1)	2 (1.9)	4 (1.3)
CC78	TGATTACA	2 (2.2)	11 (10.6)	10 (9.5)	23 (7.7)
CC8	TGATACCA/ TGATAACA	5 (5.5)	2 (1.9)	6 (5.7)	13 (4.3)
CC80	CGGTACCA			1 (1)	1 (0.3)
CC9	TGATAACT	1 (1.1)		3 (2.9)	4 (1.3)
ST101, ST106, ST7, ST154, ST96, ST725 (CC121), ST629, ST203 (CC1)	TGATTCCA	3 (3.3)	2 (1.9)	7 (6.7)	12 (4)
ST351, ST52, ST182, ST356, ST138, ST625, ST49, ST617 (CC45), ST522, ST611, ST643, ST151, ST104, ST454	TGGTTCCA		1 (1)	2 (1.9)	3 (1)
ST583 (CC80), ST685 (CC8)	TGGTACCA	1 (1.1)		1 (1)	2 (0.7)
ST67, ST198, ST395, ST426, ST479, ST66, ST68, ST520	CGGATCTA	1 (1.1)			1 (0.3)
ST93	TGGTTCTAG	4 (4.4)	2 (1.9)	3 (2.9)	9 (3)
Total		**91 (100)**	**104 (100)**	**105 (100)**	**300 (100)**

Table 3: SNP and binary types of *tst* positive isolates

MLST correlation	Profile	Binary gene subtype [a]	1989	1996	2003	Total
CC30	TGGATCCA	01000	2	3	3	8
		00000		1	2	3
CC45	CGGATCCA	01100		1		1
		01000			1	1
CC72	CGATTCCA	00100		1		1
CC8	TGATACCA	00001			1	1
		00100			1	1
ST67, ST198, ST395, ST426, ST479, ST66, ST68, ST520	CGGATCTA	01100	1			1
ST93	TGGTTCTAG	01000			1	1
Total			**3**	**6**	**9**	**18**

[a] *pvl/ cna/ sdrE/* puB110/ pT181