Epidemiological typing of meticillin-resistant *Staphylococcus aureus* isolates from Pakistan and India

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The levels of meticillin-resistant *Staphylococcus aureus* (MRSA) in Pakistan and India are known to be high, but few studies have described the epidemiology of the different MRSA clones present. In order to gain an understanding of the epidemiology of MRSA within this region, 60 MRSA isolates from Pakistan (49) and India (11) were genotyped. All isolates were typed using PFGE, staphylococcal interspersed repeat units (SIRUs), a restriction–modification method and staphylococcal cassette chromosome *mec* (SCC*mec*) typing. A subset of isolates that were distinct by PFGE and SIRUs were typed using multilocus sequence typing (MLST). Clonal complex (CC) 8 was the dominant clonal complex (57/60) and was present in both Pakistan and India. Within CC8, there were 10 SIRU profiles and 24 PFGE profiles. Two SIRU profiles were present in isolates from both India and Pakistan, whilst seven were distinct for Pakistan and one for India. All PFGE profiles were distinct for each of the two countries. Thirty-four of the 57 isolates carried SCC*mec* type III/IIIa and the remainder carried type IV SCC*mec*. MLST analysis of 14 CC8 isolates with diverse SIRU and PFGE profiles showed that all were single-locus variants, with nine belonging to sequence type (ST) 239, three to ST8 and two to ST113. From a single hospital in Pakistan, three isolates belonged to CC30 and all were indistinguishable by PFGE and SIRUs and carried the Pantone–Valentine leukocidin gene. Thus, epidemiological typing of strains from three distinct locations in India and Pakistan revealed the predominance of one clonal complex and highly related STs. The ability of SIRUs and PFGE to differentiate within ST239 demonstrates their utility in defining local epidemiology in these countries.

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INTRODUCTION

The distribution of meticillin-resistant *Staphylococcus aureus* (MRSA) is worldwide, but the frequency varies among different countries. Rates of MRSA in the subcontinent countries of Pakistan and India have been shown to be high when compared with rates in northern Europe (Anwar *et al.*, 2004; Arakere *et al.*, 2005; Stefani & Varaldo, 2003). The prevalence of meticillin resistance in *S. aureus* in Pakistan has been reported to range from 42 to

51 %, increasing from the 1990s to 2000 (Butt *et al.*, 2004; Hafiz *et al.*, 2002; Perwaiz *et al.*, 2007). Rates in India are comparable, with resistance in clinical isolates in two studies being 51.6 and 54.9 %, respectively (Anupurba *et al.*, 2003; Vidhani *et al.*, 2001).

There are very limited epidemiological typing data from these two countries, despite having a rapidly developing hospital system and a combined population of approximately 1.2 billion. Multilocus sequence typing (MLST), which examines the slowly evolving genomic core, is useful for defining ancestry and, when coupled with staphylococcal cassette chromosome *mec* (SCC*mec*) typing, is used to discriminate between different clones of MRSA. SCC*mec* typing characterizes SCC*mec* elements based on their structural differences (Oliveira & de Lencastre, 2002).

Abbreviations: CC, clonal complex; MLST, multilocus sequence typing; MRSA, meticillin-resistant *Staphylococcus aureus*; PIMS, Pakistan Institute of Medical Sciences; PVL, Pantone–Valentine leukocidin; RM, restriction–modification; SCC*mec*, staphylococcal cassette chromosome *mec*; SIRU, staphylococcal interspersed repeat unit; ST, sequence type; VNTR, variable number tandem repeat.

MLST and *SCCmec* typing were used in two separate studies to examine a small number of isolates from the same centre in Vellore, southern India. Both studies found a predominance of sequence type (ST) 239 (four out of five, and six out of six, respectively), which is mirrored across other Asian countries (Chongtrakool *et al.*, 2006; Ko *et al.*, 2005). A further study, which MLST typed five isolates from various centres across India, found that four out of five belonged to ST239 (Nadig *et al.*, 2006). The ST239 isolates that have been identified in India to date are all associated with *SCCmec* type III/IIIa. No MLST or *SCCmec* data on MRSA isolates from Pakistan are available.

Other molecular methods enabling a more detailed examination of the spread of MRSA within Pakistan and India have not been widely used. PFGE, which is highly discriminatory and useful for defining hospital outbreaks,

was used in a study of 82 MRSA isolates from Bangalore, India, demonstrating considerable diversity among the isolates, but they all clustered into three dominant clones that were shown to be genetically related to the Hungarian and Brazilian epidemic MRSA clones (Arakere *et al.*, 2005). Whilst PFGE has a high discriminatory ability, it has limitations in terms of standardization, does not reliably reveal underlying genetic relationships, and is slow and labour-intensive. Variable number tandem repeat (VNTR) typing provides a method that is both highly discriminatory and identifies the clonal origin of isolates, giving excellent correlation with MLST (Hardy *et al.*, 2006).

A recent study to determine the most prevalent MLST in southern China and Thailand showed that ST239 accounted for at least 90 % of 132 isolates (Feil *et al.*, 2008). The author postulated that ST239 is the dominant MLST of MRSA in Asia. The aim of our study was to

Fig. 1. Twenty-five PFGE profiles with associated SIRU profiles, CCs, *SCCmec* types and representative STs. * Denotes SIRU profile and *SCCmec* for the corresponding isolate.

investigate the molecular epidemiology of MRSA within Pakistan and India and to ascertain the prevalence of ST239.

METHODS

Strains. A total of 60 non-duplicate, clinical MRSA isolates were collected from three tertiary care hospitals in Pakistan [Army Medical College, Rawalpindi, $n=26$; Pakistan Institute of Medical Sciences (PIMS), $n=22$; and Kahota Research Laboratory, Islamabad, $n=1$] and one in India (Sir Ganga Ram Hospital, New Delhi, $n=11$). The MRSA strains were isolated from a variety of clinical sites: wound infections ($n=37$), respiratory isolates ($n=10$), intravenous devices ($n=9$), cerebrospinal fluid ($n=2$) and others ($n=2$).

All isolates were identified as *S. aureus* using Gram staining and catalase, coagulase and DNase tests. Meticillin resistance was confirmed using disc diffusion susceptibility testing. The antibiotic phenotype of all isolates was determined using the disc diameter breakpoints of 13 selected antibiotics active against staphylococci (British Society for Antimicrobial Chemotherapy, 2009).

MICs for vancomycin were determined using E-tests as described by Walsh *et al.* (2001). Vancomycin E-tests (0.016 – $256 \mu\text{g ml}^{-1}$; bioMérieux) were applied to inoculated brain–heart infusion agar plates and incubated for 48 h at 36°C . The following interpretive criteria were used to identify heterogeneous vancomycin-intermediate *S. aureus*: MIC $\leq 4 \mu\text{g ml}^{-1}$, sensitive; >4 to $\leq 8 \mu\text{g ml}^{-1}$, intermediate; $>8 \mu\text{g ml}^{-1}$, resistant (British Society for Antimicrobial Chemotherapy, 2009).

Molecular typing. DNA was extracted as described previously (Kumari *et al.*, 1997) for use in staphylococcal interspersed repeat unit (SIRU) typing using the method of Hardy *et al.* (2004). Primers were redesigned for locus 16 as 5'-TCAGCTTGACTCTT-CTTTCG-3' and 5'-AAGAACGTGTTGAAGGATTTT-3'. A seven-digit VNTR profile was obtained for each isolate, and isolates were

considered to be unique if they differed in repeat number at any of the seven loci (Hardy *et al.*, 2006). Primers amplifying the *lukS/F*-PV gene were used to confer potential expression of Panton–Valentine leukocidin (PVL) toxin using a modified duplex PCR assay (Lina *et al.*, 1999) with staphylococcus genus-specific 16S rRNA gene as an internal control (Monday & Bohach, 1999). PCR products were analysed using a 1.2% agarose gel run at 150 V for 2 h and stained in ethidium bromide.

Isolates for PFGE were prepared in accordance with the standardized Harmony protocol (Murchan *et al.*, 2003) using *Sma*I restriction enzyme (Invitrogen). Gel images were analysed using BioNumerics (Applied Maths), applying the Dice coefficient, with 0.5% optimization (Struelens *et al.*, 1992) and 1.9% tolerance. Isolates were considered different if they had more than one band difference.

A restriction–modification (RM) test was used to assign the isolates to one of six clonal complexes (CCs) associated with hospital-acquired MRSA. It was carried out as described previously by Cockfield *et al.* (2007). SCCmec typing was performed in accordance with the protocol of Oliveira & de Lencastre (2002).

MLST was used to characterize a subset of isolates. A representative isolate for MLST was selected from each SIRU profile and SCCmec combination. Of the 15 isolates typed, two were from India (SGRH) and 13 from Pakistan (four from the PMS and nine from the Army Medical College). Approximately 450–500 bp segments of the open reading frames of seven housekeeping genes were sequenced (Enright *et al.*, 2000). The nucleotide sequence obtained for each of the housekeeping genes was assigned a distinct allele number using the MLST database (<http://www.mlst.net/>).

RESULTS AND DISCUSSION

All isolates were typable by PFGE, SIRUs, SCCmec typing and RM assignment. Fifty-seven of the 60 isolates were closely related by all methods, all belonging to CC8, and

Fig. 2. Minimum spanning tree showing the relatedness of all isolates by SIRUs. The segments within the circles represent the proportion of isolates from each country present with the specific SIRU profile: black, Pakistan; grey, India. The distance between clusters is proportional to the number of loci at which there is a change in repeat number. SIRU profiles are shown outside the groups. ST and SCCmec type (roman numerals) are written within the SIRU clusters; the number of isolates is indicated in parentheses. *All of these isolates were from a single hospital (PIMS).

clustered with 67 % relatedness by PFGE. The remaining three isolates all belonged to CC30 (Fig. 1).

Within CC8, SIRU and PFGE typing subdivided the isolates into 10 and 24 different profiles, respectively. Variation in SIRU typing was seen mainly at loci 1, 7 and 21, and predominantly by a single repeat. Two of the SIRU profiles were present in isolates from both India and Pakistan, whilst seven were distinct to Pakistan and one to India (Fig. 2). This differed with PFGE where all profiles were distinct between the two countries. The greater number of profiles observed and the lack of common profiles between the two countries was not surprising due to the discriminatory nature and the ease with which a one-band change can occur with PFGE. When interpreting PFGE data, strains with a high degree of similarity are often considered part of the same cluster (Tenover *et al.*, 1995).

SIRU profile 2113722 was predominant among the strains from both India (seven) and Pakistan (five) and could be subdivided by PFGE into three profiles (C, M and O) that had a similarity of >75 % (Table 1). Conversely, one PFGE profile (N) could be subdivided using SIRUs. The most common SIRU profile (3113822) from Pakistan was a cluster of 11 isolates from a single hospital (PIMS), all of which had indistinguishable PFGE profiles. These data implied that this was either a predominant local strain or that these isolates were part of an outbreak. Although isolates were sequential non-duplicate isolates, no epidemiological data were available.

SCCmec typing identified three different SCCmec types, types III ($n=22$), IIIa ($n=12$) and IV ($n=26$). Only SCCmec type III and subtype IIIa were identified in the isolates from India (four and seven, respectively). All the type IIIa isolates from India were confined to a single SIRU group (2113722), with the same SIRU profile being present in Pakistan but with SCCmec type III.

MLST typing of a representation of the strains within CC8 with diverse SIRU and PFGE patterns revealed that the isolates belonged to three STs, ST239 (nine), ST8 (three) and ST113 (two). ST239 and ST113 are single-locus variants of ST8, the predicted clonal ancestor of CC8. All the ST239 strains were SCCmec type III or IIIa, and if it is postulated that other strains that were not MLST typed but had the same SIRU profile and SCCmec type were ST239, this would account for 34 strains. ST239 was present in strains from both India and Pakistan. The worldwide dissemination of ST239 (Brazilian/Hungarian clone) is attributed to its evolution from the highly successful and unrelated lineages of CC8 (ST8) and CC30 (ST30), with some studies associating it with an increase in virulence (Amaral *et al.*, 2005; Edgeworth *et al.*, 2007). Although the exact mechanism of DNA transfer is unknown, it has been postulated that fusion of the two *S. aureus* strains may have occurred (Lindsay, 2008; Robinson & Enright, 2004). All the strains of ST8 or ST113 carried SCCmec type IV. Within one SIRU profile where differing PFGE profiles were observed, all three different ST types were identified.

This presence may be attributed to local clonal evolution. Polymorphisms may have occurred within ST8-SCCmecIV to evolve into ST113-SCCmecIV or vice versa, or the strains may have arisen through multiple insertions of SCCmec (Nübel *et al.*, 2008).

The limited number of STs present within these countries demonstrates the importance of using a typing technique with sufficient discriminatory ability to enable investigations of the transmission and spread of MRSA to be undertaken. VNTRs have been shown to provide a more stable means of investigating transmission and evolution events than PFGE (Sola *et al.*, 2003).

The three CC30 isolates were all from the same hospital (PIMS) in Pakistan and were identical by SIRUs and PFGE with SCCmec IV and all carried the PVL toxin gene. MLST typing of one isolate demonstrated that it was ST30. They were isolated from pus ($n=2$) and an intravenous device ($n=1$). ST30 is a South West Pacific community MRSA clone predominant in Europe and the South West Pacific Islands and is characterized by the presence of SCCmec types IV and V and the PVL gene (Smith & Cook, 2005; Udo *et al.*, 2008; Vandenesch *et al.*, 2003). Although not observed previously in Pakistan, it has been described in Singapore, China and Kuwait (Ho *et al.*, 2004; Hsu *et al.*, 2006; Udo *et al.*, 2008).

Many of the isolates tested were resistant to multiple antibiotics. One isolate (In4) was found to have heterogeneous resistance to vancomycin with an MIC of $6 \mu\text{g ml}^{-1}$. However, 46 of the isolates had MICs $\geq 2 \mu\text{g ml}^{-1}$, which has been noted previously among ST239 isolates from Singapore (Hsu *et al.*, 2007). Resistance to mupirocin (2 %) was lower than in a previous report from a group in India where 6 % of isolates from 200 clinical isolates were resistant to mupirocin (Gadepalli *et al.*, 2007). The low levels of resistance to mupirocin may be due to its limited usage. In contrast, 50 % of isolates were rifampicin resistant, a much higher rate than reported in other countries, e.g. 2.2 % in the UK (Bernard *et al.*, 2008; Gould *et al.*, 2008). Given the high prevalence of tuberculosis in these countries (World Health Organization tuberculosis fact sheets available at http://www.who.int/tb/publications/global_report/2009/pdf/pak.pdf and http://www.who.int/tb/publications/global_report/2009/pdf/ind.pdf), the use of rifampicin, a first-line anti-tuberculosis agent, may account for this increased resistance. Sekiguchi *et al.* (2006) had similar findings, with 94 % of MRSA isolates being resistant to rifampicin on a tuberculosis ward in Tokyo.

In conclusion, epidemiological typing of strains from three distinct locations in India and Pakistan revealed that there is one predominant ST type, ST239. The ability of SIRUs and PFGE to differentiate within ST239 demonstrates their utility in defining local epidemiology. SIRUs could also be used for defining the spread of this important clone and its subtypes both within and between countries.

Table 1. Characteristics of isolates

SIRU group	SIRU profile	Isolate	PFGE group	RM*	MLST (ST)	SCCmec type	MIC (μg ml ⁻¹)†	Antibiogram‡												
								Van	Van	Mup	Cip	Ery	Neo	Gen	Ami	Tet	Tig	Chlo	Rif	Lin
1	2113622	PK26	A	CC8	ST239	III	2	S	S	R	R	S	R	R	R	S	S	R	S	S
		PK27	A	CC8		III	3	S	S	R	R	S	R	R	R	S	S	R	S	S
		PK28	B	CC8		III	1.5	S	S	R	R	S	R	R	R	S	S	R	S	S
		PK39	D	CC8		III	1	S	S	R	R	S	R	R	R	S	S	R	S	R
		PK40	D	CC8		III	2	S	S	R	S	S	R	R	R	S	S	R	S	S
		PK42	W	CC8		III	1.5	S	S	R	S	S	R	R	R	S	S	R	S	S
		In4	Q	CC8		III	6	S	S	R	R	R	R	R	R	S	S	R	S	S
		In7	R	CC8		III	2	S	S	R	R	R	R	R	R	S	S	R	S	S
2	2113722	PK11	C	CC8	ST239	III	2	S	S	R	S	S	R	R	R	S	S	R	S	S
		PK21	C	CC8		III	2	S	S	R	S	S	R	R	R	S	S	R	S	S
		PK23	C	CC8		III	2	S	S	R	S	S	R	R	R	S	S	R	S	S
		PK7	C	CC8		III	2	S	S	R	S	S	R	R	R	S	S	R	S	S
		PK9	C	CC8		III	2	S	S	R	S	S	R	R	R	S	S	R	S	S
		In1	O	CC8		IIIa	3	S	S	R	R	R	R	R	R	S	S	R	S	S
		In2	O	CC8		IIIa	2	S	S	R	R	R	R	R	R	S	S	R	S	S
		In3	O	CC8		IIIa	3	S	S	R	R	R	R	R	R	S	S	R	S	S
		In8	O	CC8		IIIa	4	S	S	R	R	R	R	R	R	S	S	R	S	S
		In11	M	CC8		IIIa	2	S	S	R	R	R	R	R	R	S	S	R	S	S
		In5	M	CC8		IIIa	3	S	S	R	R	R	R	R	R	S	S	R	S	S
		In6	M	CC8		IIIa	3	S	S	R	R	R	R	R	R	S	S	R	S	S
3	2113822	PK31	S	CC8	ST239	III	2	S	S	R	R	R	R	R	R	R	S	S	S	S
		PK32	N	CC8		III	1.5	S	S	R	R	R	R	R	R	S	S	R	S	S
		PK37	X	CC8		III	0.5	S	S	R	R	R	R	R	R	R	S	S	S	S
		PK38	X	CC8		III	1.5	S	R	S	S	R	R	S	S	R	S	S	S	S
		PK57	X	CC8		III	3	S	S	S	R	S	R	S	S	S	R	R	S	R
4	3113522	PK61	P	CC8	ST239	III	2	S	S	R	R	S	R	R	R	S	S	R	S	S
5	3113722	In9	J	CC8	ST239	III	2	S	S	R	R	S	S	S	R	S	S	R	S	S
		In10	J	CC8		III	2	S	S	R	R	S	S	S	R	S	S	R	S	S
6	3113822	PK17	L	CC8	ST113	IV	2	S	S	R	R	R	R	R	I	R	S	S	S	S
		PK18	L	CC8		IV	4	S	S	R	R	R	R	R	I	R	S	S	S	S
		PK19	L	CC8		IV	1.5	S	S	R	R	R	R	R	I	R	S	S	S	S
		PK20	L	CC8		IV	2	S	S	R	R	R	R	R	I	R	S	S	S	S
		PK24	L	CC8		IV	2	S	S	R	R	R	R	R	I	R	S	S	S	S
		PK25	L	CC8		IV	2	S	S	R	R	R	R	R	I	R	S	S	S	S
		PK29	L	CC8		IV	3	S	S	R	R	R	R	R	R	I	R	S	S	S

Table 1. cont.

SIRU group	SIRU profile	Isolate	PFGE group	RM*	MLST (ST)	SCCmec type	MIC (µg ml ⁻¹)†	Antibiogram‡												
								Van	Van	Mup	Cip	Ery	Neo	Gen	Ami	Tet	Tig	Chlo	Rif	Lin
		PK6	L	CC8		IV	2	S	S	R	R	R	R	I	R	S	S	S	S	S
		PK30	L	CC8		IV	1	S	S	R	R	R	R	I	R	S	S	S	S	S
		PK5	L	CC8		IV	1.5	S	S	R	R	R	R	R	R	S	S	S	S	S
		PK8	L	CC8		IV	0.5	S	S	R	R	R	R	S	R	S	S	S	S	S
7	01131023	PK59	Y	CC8	ST239	III	1.5	S	S	R	R	R	R	R	R	S	S	S	S	R
8	21131023	PK58	E	CC8	ST8	IV	3	S	S	S	R	S	S	S	S	S	S	S	S	R
9	31131023	PK44	K	CC8	ST8	IV	2	S	S	S	R	S	S	S	S	S	S	S	S	R
		PK47	K	CC8		IV	1.5	S	S	S	R	S	S	S	S	S	S	S	S	R
		PK49	K	CC8		IV	2	S	S	S	R	S	S	S	S	S	S	S	S	R
		PK50	K	CC8		IV	2	S	S	S	R	S	S	S	S	S	S	S	S	R
		PK55	K	CC8		IV	3	S	S	S	R	S	S	S	S	S	S	S	S	S
10	31131022	PK36	F	CC8	ST113	IV	2	S	S	R	R	R	R	R	R	S	S	S	S	S
		PK41	F	CC8		IV	2	S	S	R	R	R	R	R	R	S	S	S	S	S
		PK48	F	CC8		IV	1.5	S	S	R	R	R	R	R	R	S	S	S	S	S
		PK46	G	CC8		IV	3	S	S	R	R	R	R	R	R	S	R	S	S	S
		PK43	H	CC8		IV	2	S	S	R	R	R	R	I	R	S	R	S	S	S
		PK45	I	CC8	ST8	IV	3	S	S	R	R	S	R	R	R	S	S	S	S	S
		PK56	N	CC8	ST239	IIIa	1.5	S	S	R	R	R	R	R	R	S	S	S	S	S
		PK54	T	CC8		IIIa	3	S	S	R	R	R	R	R	S	S	S	S	S	
		PK60	T	CC8		IIIa	2	S	S	R	R	R	R	S	R	S	S	R	S	R
		PK33	U	CC8	ST239	IIIa	2	S	S	R	R	R	R	S	R	S	S	R	S	S
		PK52	U	CC8		IIIa	3	S	S	R	R	R	R	S	R	S	S	R	S	S
11	1222932	PK2	V	CC30	ST30	IV	2	S	S	R	R	S	R	S	S	S	S	R	S	R
		PK3	V	CC30		IV	2	S	S	R	R	S	R	S	S	S	S	R	S	R
		PK12	V	CC30		IV	3	S	S	R	S	S	R	R	S	S	R	S	S	
Resistance of all 60 isolates to each antibiotic tested (%)								0	2	87	85	59	87	57 17§	83	5	5	52	0	20

*RM, Restriction–modification CC assignment.

†MICs: ≤4 µg ml⁻¹, sensitive; >4 to ≤8 µg ml⁻¹, intermediate; >8 µg ml⁻¹, resistant.

‡Van, Vancomycin (5 µg); Mup, mupirocin (20 µg); Cip, ciprofloxacin (1 µg); Ery, erythromycin (5 µg); Neo, neomycin (10 µg); Gen, gentamicin (10 µg); Ami, amikacin (30 µg); Tet, tetracycline (10 µg); Tig, tigecycline (15 µg); Chlo, chloramphenicol (10 µg); Rif, rifampicin (2 µg); Lin, linezolid (10 µg); FuA, fusidic acid (10 µg).

§Intermediate resistance to amikacin.

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