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Full Length Research Paper

Epidemiology of methicillin resistant *Staphylococcus aureus* (MRSA) isolates from Pakistan

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In order to have an understanding of the distribution of MRSA clones in Pakistan, where unregulated antibiotic use is widespread and the distribution of MRSA is thought to be high, an epidemiological study was designed. In this study, epidemiological relationships between 123 methicillin resistant *Staphylococcus aureus* strains, isolated between 2006 and 2008 from three tertiary care hospitals of Rawalpindi and Islamabad, were examined using six loci in a multilocus variable number tandem repeat analysis (MLVA). A total of 63 haplotypes were obtained by MLVA. Analysis of restriction modification (RM) genes detected an RM3 type, associated with CC8 in 98% of strains and an RM1 type, associated with CC30, in only two strains. On further typing of selected strains by Spa typing and MLST, it was found that the RM3/CC8 isolates were ST113-t064, ST113-t451 or ST239, with one of four spa types, while the RM1/CC30 isolates were ST30-t021. We concluded that there is a high prevalence of CC8 MRSA strains in Rawalpindi/Islamabad, Pakistan and frequent exchange of strains between closely-linked hospitals.

Key words: Epidemiology, MRSA, MLVA, MLST, Pakistan, spa.

INTRODUCTION

Staphylococcus aureus is a versatile and dangerous human bacterial pathogen. It is a common cause of nosocomial and community acquired infections (Steinberg et al., 1996; Lowy, 1998). *S. aureus* has developed resistance to the antibiotic 'methicillin' and continued spread of methicillin-resistant *S. aureus* (MRSA) strains poses a significant risk to patients and contributes to a substantial financial burden on healthcare resources (Speller et al., 1997; Kim et al., 2001). Geographic spread of one or several MRSA clones has been reported and proven by molecular epidemiology (de Lencastre et al., 1997; de Souza et al.,1998; Aucken et al., 2002). MRSA isolates usually belong to six lineages (CC1, CC5, CC8, CC22, CC30 and CC45) out of ten dominant clonal complexes (CCs) or lineages. The major lineages found in UK and European hospitals are CC22, CC30 and CC8, CC5,CC30 respectively (Enright et al., 2000; de Sousa and de Lencastre, 2003; Roth et al., 2004; Ghebremendhin et al., 2005), while hospitals in the USA are dominated by CC5, CC8, CC30 and CC45 (McDougal et al., 2003). In Asia, CC5 strains are prevalent in Korea and Japan with CC8 in other Asian countries (Ko et al., 2005; Song et al., 2011). In order to perform molecular epidemiology of MRSA isolates from Pakistan, we focused on clinical MRSA isolates from three tertiary care hospital located in Rawalpindi/Islamabad and examined the types and phylogenetic relationship of the isolates. In the current study, an MLVA scheme was combined with RM typing, spa typing and MLST to investigate the phylogeny of the Pakistani MRSA isolates.

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Abbreviations: MLST, Multi locus sequence typing; **MLVA,** multilocus variable number of tandem repeat analysis; **MT,** MLVA type; **ST,** sequence type; **CC,** clonal complex; **RM,** restriction modification.

Table 1. List of primers used for MLVA typing of *S. aureus* MRSA.

Gene	Primer sequences	Repeat length (bp)	Label[1]	*Tm* (°C)
clfA (Clumping factor A)	GCATTTAATAACGGATCAGG TGAATTAGGCGGAACTACAT	18	FAM	58.5
clfB (Clumping factor A)	ATGGTGATTCAGCAGTAAATCC CATTATTTGGTGGTGTAACTCTT	18	Vic	61.5
sdrC (Ser-Asp-rich fibrinogen binding proteins)	ATGATTTCACACTTGATAATGGC GCTGTTTTATGCTGATCTTTAAC	18	Vic	61.5
sdrD (Ser-Asp-rich fibrinogen binding proteins)	ATGATTTCACACTTGATAATGGC TAACGTTGCGTTATTTGAGCCC	18	Tet	61.5
Spa (Protein A)	AGCACCAAAAGAGGAAGACAA GTTTAACGACATGTACTCCGT	24	FAM	62
Sspa (Serine protease V8)	ATCMATTTYGCMAAYGATGACCA TTGTCTGAATTATTGTTATCGCC	18	Ned	61.5

[1]Flourescent dyes as indicated were present in the forward primer.

MATERIALS AND METHODS

Bacterial strains and isolates

A collection of methicillin resistant *S. aureus* strains were isolated from three tertiary care hospitals of Islamabad/Rawalpindi during the years 2006 to 2008. Of the 123 strains, 5 were isolated from blood, 27 from catheter tips, 7 from ear infections, 54 from pus, 17 from sputum, 3 from spinal cord, 4 from tissue fluids and 6 from urine. All these strains were stored at -80°C in a 20% glycerol solution. Strains NCTC 8325, Mu50, MRSA252 and CDC8 were used as controls.

Bacterial culture and DNA extraction

MRSA strains stored at -80°C were streaked onto BHI agar (Oxoid) and cultured over night at 37°C. The colonies were visually inspected and then a single colony was picked and inoculated unto BHI broth (Oxoid) and incubated over night (16 to 24 h) in a shaking incubator. 1.5 ml of this fresh culture was taken and centrifuged at 10,000 rpm for 10 min. The pellet was re-suspended in 250 µl P-1 buffer (Qiagen) and to this were added 2.5 µl (100 µg/ml) lysostaphin, 2 µl (25 mg/ml) proteinase K, mixed and 27 µl 10% SDS. Tubes were inverted 3 to 4 times and incubated at 37°C for 20 to 30 min. Then, 98 µl 5 M NaCl and 81 µl preheated CTAB (incubated CTAB for 20 min at 65°C) was added. After mixing, an equal volume of 24:1 chloroform : isoamyl alcohol was added and tubes were centrifuged at 10,000 rpm for 10 min. The upper layer was transferred to a fresh tube and an equal volume of isopropanol was added prior to centrifugation at 10,000 rpm for 10 min and resuspenion of the DNA pellet in 50 µl sterile TE buffer pH 8.

Multilocus variable number of tandem repeat analysis (MLVA)

Six genes were selected for the multiplex PCR for typing of MRSA strains by MLVA: *clfA* (clumping factor A), *clfB* (clumping factor B), *sdrC* (Ser-Asp-rich fibrinogen binding proteins), *sdrD* (Ser-Asp-rich fibrinogen binding proteins), *spa* and *sspa.* The choice of VNTR was based on established MLVA protocols (Sabat et al., 2003; Francois et al., 2005; Gilbert et al., 2006) and available genome sequence data (http://www.ncbi. nlm.nih.gov /genomes/prokes.cgi). Pairs of primers, one of which was fluorescently labeled, were used to amplify each gene (Table 1). Primer sequences were the same as used in previous studies (Harmsen et al., 2003; Sabat et al., 2003). The genes *clfA*, *sdrC* were amplified in a multiplex PCR reaction separately from *clfB*, *sdrD*, *spa* and *sspa*. The reaction mixture contained 2 µl (40 ng) of genomic DNA, 2.5 µl 10X PCR buffer (MBI Fermentas, UK), 1.5 µl 25 mM $MgCl_2$, 0.8 µl 10 mM dNTPs, 1.5 µl 5 µM each primer and one unit of Taq DNA polymerase (Fermentas, UK) and 9 µl PCR grade water. The reaction mixture was taken through thermocycling conditions consisting of 5 min of 94°C followed by 30 cycles of amplification each consisting of three steps: thirty seconds at 94°C; thirty seconds at 53°C and two minutes at 72°C, followed by a final extension step of 5 min at 72°C. PCR was performed using a Triple Master Mix Thermocycler (Eppendorf, USA). The amplified products were subjected to gene scan for size analysis in ABI-sequencer.

Gene scan protocol

PCR products were diluted 1:10 with sterile distilled water. Then, 0.5 µl of diluted (1:10) sample was added to 0.5 µl Liz1200 (ABI) size standard and 9 µl of formamide before heating to 95°C for 3 min. Samples were electrophoresed on an ABI DNA Sequencer. Gene scan peaks were analyzed using the Applied Biosystem Peak Scanner software volume 1.0 to obtain the exact PCR product size (http:// www. Applied bio systems.com/absite/us/home/support/software-community/free-ab-software. html.

Calculation of VNTRs and MLVA

The length of the tandem repeats and flanking regions were calculated for all the loci from the standard sequenced strains NCTC8325 and Mu50 using the Tandem Repeats Finder Program, Version 4.03 (Benson, 1999). These sizes were confirmed by sequencing the seven loci from two Pakistani isolates (P.18431 and A.14256). These sizes were used to calculate the number of

repeats present in the PCR products obtained from the Pakistani MRSA isolates. The formula used to calculate the number of repeats was: size of the PCR product minus size of the flanking region divided by the size of the repeat unit.

Analysis of lineages by typing of restriction modification genes

The analysis of RM genes was performed as described by Cockfield et al. (2007). Briefly, two *hsdS* genes, *sau1hsdS1* and *sau1hsdS2*, were amplified using different combinations of primers specific for the constant and variable regions of these genes. This involved performing three PCR reactions designed to separate strains into an RM1, RM2 or RM3 type. Strains of a known RM type were utilized as controls.

Detection of SCCmec

SCCmec typing was performed in a multiplex PCR as described by Boye et al. (2007).

Spa typing and multilocus sequence typing (MLST)

Spa typing was performed as described by Harmsen et al. (2003). The 24 bp repeat units were fed into the ridom spa server and repeat codes were identified and entered into the ridom spa server to obtain the spa types (www.ridom.de/staphtype).

The *S. aureus* MLST typing was performed as described by Enright et al. (2000). The allelic profile of *S. aureus* strains were obtained by sequencing internal fragments and querying these sequences against the MLST server (http://saureus.mlst.net) to define the alleles, allelic profile and ST type.

Phylogenetic analysis

The number of tandem repeats obtained for all the loci for each strain were imported into Bionumeric (Applied Maths Inc), in order to calculate a phylogenetic tree. In the trees (Figure 1), each circle represents a specific MLVA type (the MT type/haplotype) and the size and color of the circle represents the number of strains in this specific type and their source of isolation. Isolates varying in repeat number at one locus are shown connected by a line and are clustered together.

RESULTS

In the current study, we focused on clinical MRSA isolates from three tertiary care hospitals located in the twin cities of Rawalpindi/Islamabad and examined the types and phylogenetic relationship of the isolates. A total of 123 nosocomial MRSA isolates were obtained: 89 isolates from Hospital P, a large tertiary care hospital in Islamabad; 7 isolates from Hospital K, a medium-size hospital in Islamabad; and 27 isolates from Hospital A, a large unit in Rawalpindi. Isolates were obtained from a range of infection types/sources and were from different individuals.

An MVLA approach was adapted to the analysis of these strains as a rapid and cost-effective method of establishing their phylogenetic relationships. PCR products were analyzed using an ABI sequencer as this method of MLVA typing is more accurate and robust as compared to analyzing fragments on gels due to its ability to separate PCR amplicons with small differences in size thereby providing accurate sizes for these products. All the isolates from Pakistan yielded PCR products with all the VNTR loci. PCR products of the expected sizes, as predicted from analysis of genome sequences, were obtained for the control strains NCTC 8325 and Mu50. The PCR products sizes were then converted into repeat numbers. The length of flanking region and number of repeats were calculated using two sequenced strains, NCTC8325 and Mu50, as controls and sequences for six of the loci derived from isolates P.18431 and A.14256. In addition, we noted that the expected repeat numbers were found in 26 isolates sequenced for spa typing. Some VNTR loci contained large numbers of repeats, e.g. *clfA* contained up to 46 repeats and *clfB* up to 55, while some had limited number of repeats, *sspa* had four or five repeats.

The number of repeats calculated for the six loci for all the isolates are given in Figure 3 in the form of an allelic profile. A total of 63 MT (genotypes/haplotypes) were obtained for the Pakistani isolates. Two large clusters of 10 and 11 identical isolates (MTs 13 and 47) were detected with all these isolates being derived from pus and Hospital P. Smaller clusters of isolates with a common and unusual source were also detected. Thus, MT17 consisted of five blood isolates (November 2007 to February 2008), MT25 of six urine isolates (April to July 2006) and MT30 of three spinal cord isolates (August 2006/April 2007). These isolates are indicative of clusters of MRSA infection cases.

MLVA profiles were clustered into minimum spanning trees using Bionumerics in order to show the genetic relationships among the various MLVA types (Figure 1). In the tree, repeat numbers were treated as categories. Thus, two MTs differing in repeats at one locus are clustered together and connected by bold line while the isolates varying in more than two loci are connected by a thin line, while the standard strains are connected by dotted lines.

In order to establish the clonal relationship of these isolates to other global collections of MRSA isolates, further analyses were carried out. Firstly, we investigated the clonal relationships of these isolates by analyzing the RM systems as described by Cockfield et al. (2007). This showed that 121 isolates were positive for an RM3 type (Figure 2) and only two strains, P.1286 and P.16809, for an RM1 type (data not shown). This indicated that the majority of isolates belonged to CC8, which is associated with an RM3 type. Then, spa typing and MLST were performed on a sub-group of strains (Table 2). Spa typing was performed on 26 isolates which also included multiple isolates from the same MT (e.g. five were from MT13). All isolates from the same MT had identical spa types indicating that MLVA grouped highly-related

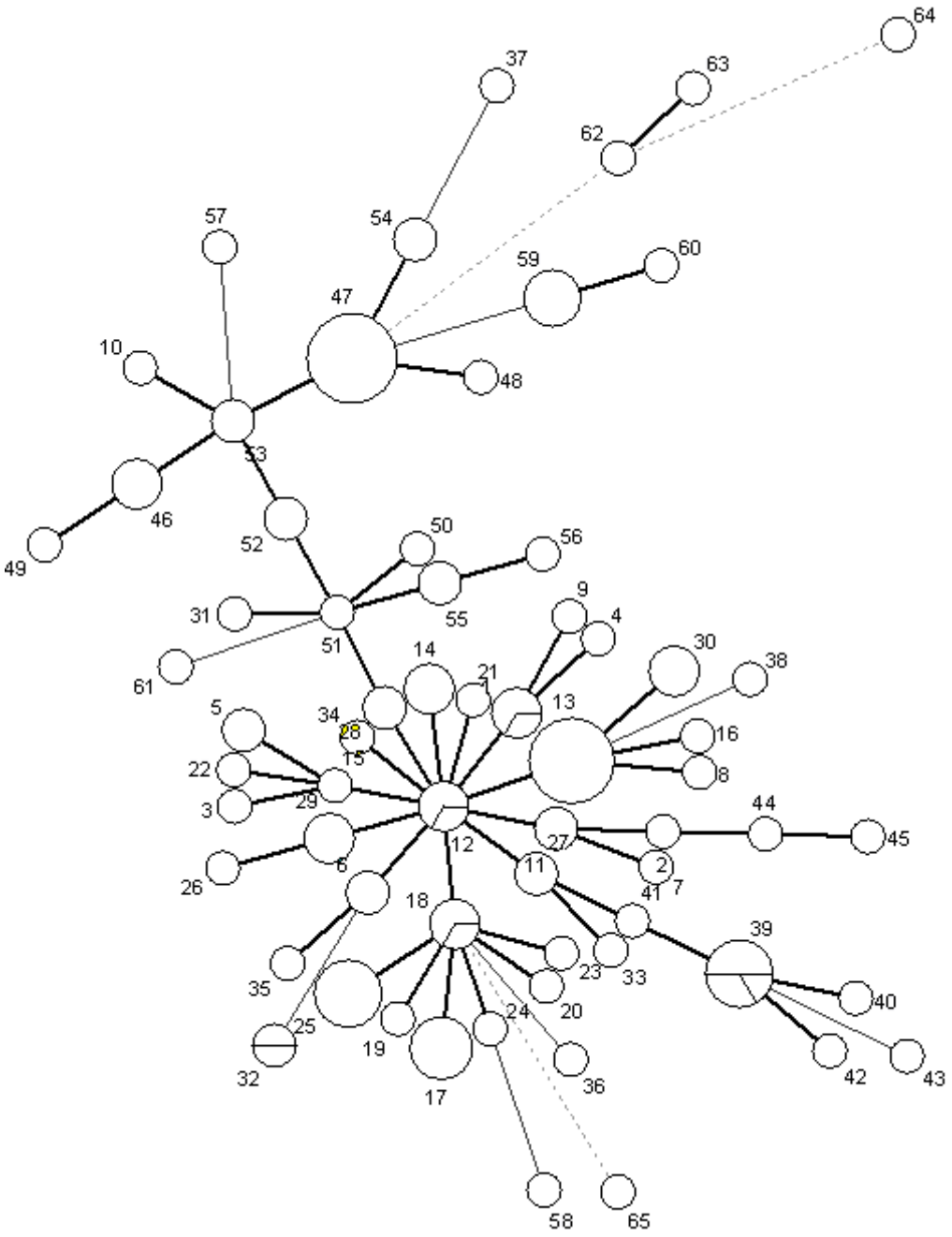


Figure 1. Minimum spanning tree of Pakistani MRSA isolates derived from VNTRs for *clfA*, *clfB*, *sdrD*, *sdrC*, *spa* and *sspa* loci using Bionumerics software. Circle sizes are proportional to number of MTs in each node. Isolates varying in repeat number at a single locus are connected with bold lines, while isolates varying at two or more loci are connected with thin lines. Control strains are connected by dotted lines while the divided nodes show the presence of isolates from different sources.

strains, while the separation of isolates having the same spa type into different MTs indicates the presence of genetic diversity within a spa type. The MLVA scheme used in the typing of Pakistani MRSA was the same as used by Sabat et al. (2003) but differed in that fluorescently labeled primers in two multiplex PCR were employed in order to get exact PCR product sizes by analysis on a automated DNA sequencer. This approach provided a high degree of accuracy to the fine typing of MRSA at a sub species level.

There was a wide distribution of spa types within these three Pakistan hospitals. Thus, among the 16 isolates

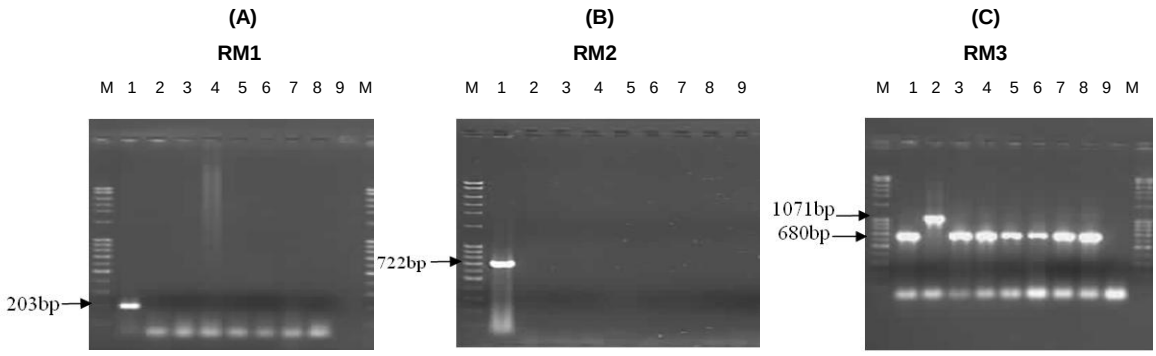


Figure 2. RM tests with Pakistani *S. aureus* MRSA isolates. This figure shows the amplicons obtained from three RM test with Pakistani *S. aureus* isolates: RM1 (panel A); RM2 (panel B); and RM3 (panel C). Panel A: M, 100 bp DNA ladder (Norgen Full Ranger); lanes 1 to 9 contain MRSA252 [Positive control for RM1], NCTC8325, P.18431, A.14256, K.3, P.2113, P.2112, A.26163 and a negative control, respectively. Panel B: lanes 1 to 9 contain CDC-8 [Positive control for RM2], NCTC8325, P.18431, A.14256, K.3, P.2113, P.2112, A.26163 and a negative control. Panel C: lanes 1 to 9 contain NCTC8325, Mu50 [Positive controls for RM3], P.18431, A.14256, K.3, P.2113, P.2112, A.26163 and a negative control. All the Pakistani MRSA isolates showing positive results within an RM3 test giving a PCR product size of 680 bp, indicative of CC8, while Mu50 has a 1071 bp amplicon size indicative of CC5.

from hospital P which were spa typed, we found six spa types: t064 (6 isolates), t030 (4 isolates), t632 (2 isolates), t987 (2 isolates), t451 (1 isolate) and t021 (1 isolate) and among the 9 isolates from Hospital A, we found four spa types: t064 (1 isolate), t275 (2 isolates), t632 (2 isolates) and t037 (1 isolate). MLST was carried out for 10 isolates representing different spa types. Out of 5 isolates from hospital A, we found one ST113 and four ST239, while out of 5 from Hospital P, three were ST113, one was ST239 and one was ST30 (Table 2). Thus, nine out-of-ten isolates had MLST types associated with CC8 and one with CC30.

DISCUSSION

In this study, MLVA typing was used as a robust, cost effective and discriminatory method for understanding the distribution and phylogenetic relationships of MRSA isolates in twin cities (Islamabad/Rawalpindi) of Pakistan. Further analysis by an RM test, spa typing and MLST helped to understand how these clones are related to the global MRSA clones.

It has been reported that VNTR typing provides greater insight into the transmission mode and evolutionary events than PFGE (Sola et al., 2003) especially when it is combined with other typing methods (Cockfield et al., 2007). MLVA with 123 MRSA isolates produced a total of 63 MT (genotypes/haplotypes) with two large clusters of 10 and 11 identical isolates (MTs 13 and 47), all these isolates being derived from pus and Hospital P. This is suggestive of a common source of infection and also of a local outbreak or predominant local strain. Some additional smaller clusters of isolates with a common source were also detected such as MT17, MT25 and MT30 (Figure 3). Other MLVA types, for example MT12, MT18, MT29 and MT39, contained isolates from different hospitals but with the same allelic profile confirming the prevalence of a common strain in more than one hospital. These isolates are indicative of clusters of MRSA infection cases.

In order to understand the genetic relationships among the various MLVA types, a minimum spanning tree was generated with Bionumerics using their MLVA profiles. In the tree, repeat numbers were treated as categories and thus two MTs differing in repeats at one locus are clustered together and connected by bold line while the isolates varying in more than two loci are connected by a thin line. The MLVA clusters were globally linked by RM typing, spa and MLST. The majority of isolates were found to be from CC8 and a few from CC30 by RM typing and MLST with the following ST types being represented: ST239, ST113 and ST30. Spa types were obtained and confirmed these findings (Table 2). This shows that some clones (CC8 and CC30) of MRSA are prevalent in this area (Islamabad/Rawalpindi) as well as in other parts of Pakistan (Zafar et al., 2011). These results are also in concordance with the studies made in other Asian countries. All other Asian countries except Korea and Japan are dominated by strains from CC8, particularly ST239 and ST8, and CC30 with ST30 (Ko et al., 2005; Song et al., 2011). The ST113 clone is a single locus variant of ST8 and may have evolved from this clone due to genetic polymorphisms or multiple insertion events (Shabir et al., 2010) as has been observed for evolution of CC5 strains (Nubel et al., 2008). The distribution of CC8 and CC30 has been reported previously in all other parts of the world but especially in Europe and USA

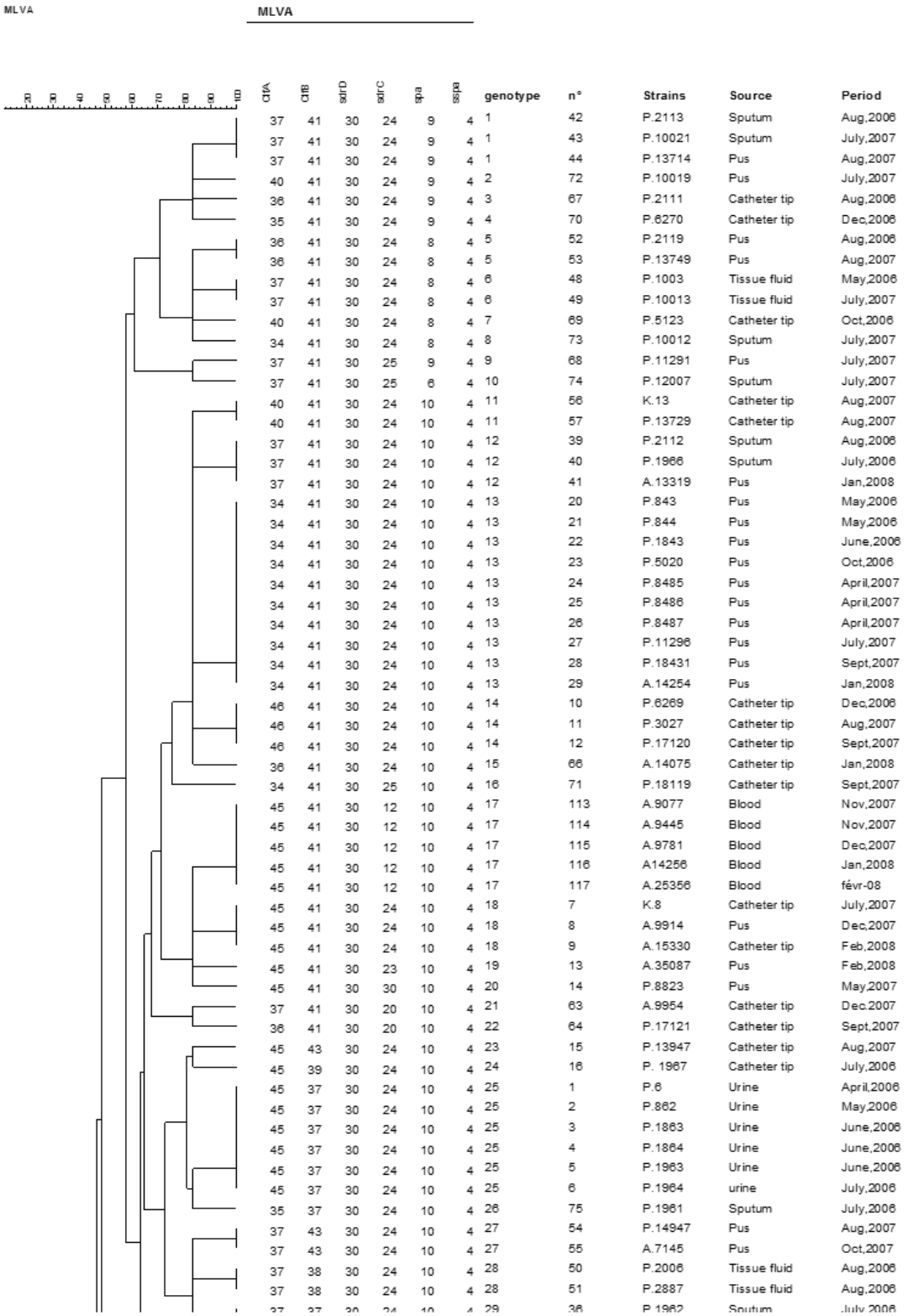
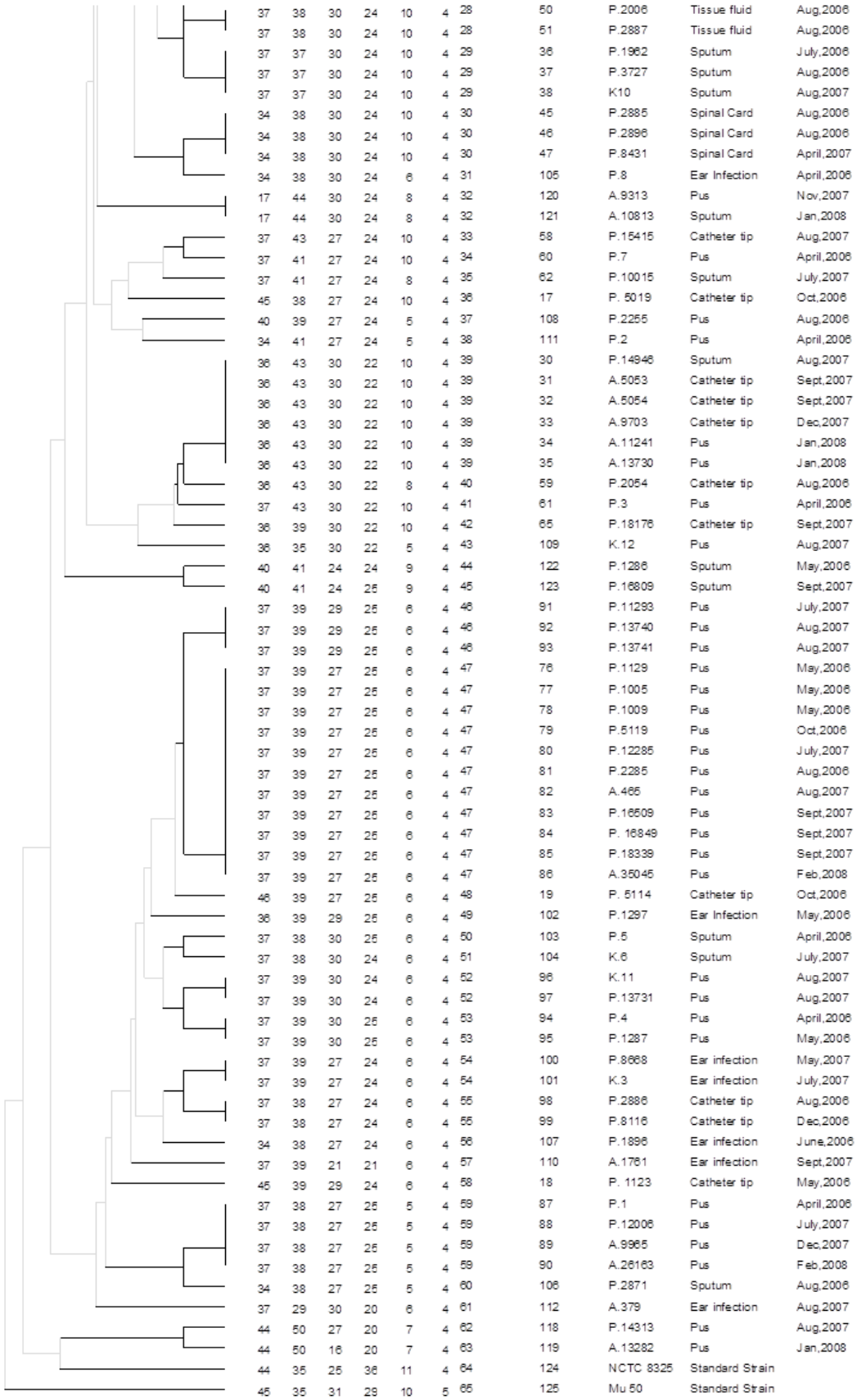


Figure 3. Dendrogram derived from the MLVA types of the 123 Pakistani MRSA isolates and two standard strains. Repeat numbers, as indicated, were derived for six genes containing variable numbers of tandem repeats. A genotype (termed MLVA type or MT in the text or haplotype) was assigned to each unique combination of repeat numbers and then these genotypes were utilized for a phylogenetic analysis in Bionumerics. The dendrogram construction method was UPGMA with a cluster cut-off of 80% and a categorical distance coefficient. Strain names, source of isolate and time of isolation are also indicated.



37	38	30	24	10	4	28	50	P.2006	Tissue fluid	Aug,2006
37	38	30	24	10	4	28	51	P.2887	Tissue fluid	Aug,2006
37	37	30	24	10	4	29	36	P.1962	Sputum	July,2006
37	37	30	24	10	4	29	37	P.3727	Sputum	Aug,2006
37	37	30	24	10	4	29	38	K10	Sputum	Aug,2007
34	38	30	24	10	4	30	45	P.2885	Spinal Card	Aug,2006
34	38	30	24	10	4	30	46	P.2896	Spinal Card	Aug,2006
34	38	30	24	10	4	30	47	P.8431	Spinal Card	April,2007
34	38	30	24	6	4	31	105	P.8	Ear Infection	April,2006
17	44	30	24	8	4	32	120	A.9313	Pus	Nov,2007
17	44	30	24	8	4	32	121	A.10813	Sputum	Jan,2008
37	43	27	24	10	4	33	58	P.15415	Catheter tip	Aug,2007
37	41	27	24	10	4	34	60	P.7	Pus	April,2006
37	41	27	24	8	4	35	62	P.10015	Sputum	July,2007
45	38	27	24	10	4	36	17	P. 5019	Catheter tip	Oct,2006
40	39	27	24	5	4	37	108	P.2255	Pus	Aug,2006
34	41	27	24	5	4	38	111	P.2	Pus	April,2006
36	43	30	22	10	4	39	30	P.14946	Sputum	Aug,2007
36	43	30	22	10	4	39	31	A.5053	Catheter tip	Sept,2007
36	43	30	22	10	4	39	32	A.5054	Catheter tip	Sept,2007
36	43	30	22	10	4	39	33	A.9703	Catheter tip	Dec,2007
36	43	30	22	10	4	39	34	A.11241	Pus	Jan,2008
36	43	30	22	10	4	39	35	A.13730	Pus	Jan,2008
36	43	30	22	8	4	40	59	P.2054	Catheter tip	Aug,2006
37	43	30	22	10	4	41	61	P.3	Pus	April,2006
36	39	30	22	10	4	42	65	P.18176	Catheter tip	Sept,2007
36	35	30	22	5	4	43	109	K.12	Pus	Aug,2007
40	41	24	24	9	4	44	122	P.1286	Sputum	May,2006
40	41	24	25	9	4	45	123	P.16809	Sputum	Sept,2007
37	39	29	25	6	4	46	91	P.11293	Pus	July,2007
37	39	29	25	6	4	46	92	P.13740	Pus	Aug,2007
37	39	29	25	6	4	46	93	P.13741	Pus	Aug,2007
37	39	27	25	6	4	47	76	P.1129	Pus	May,2006
37	39	27	25	6	4	47	77	P.1005	Pus	May,2006
37	39	27	25	6	4	47	78	P.1009	Pus	May,2006
37	39	27	25	6	4	47	79	P.5119	Pus	Oct,2006
37	39	27	25	6	4	47	80	P.12285	Pus	July,2007
37	39	27	25	6	4	47	81	P.2285	Pus	Aug,2006
37	39	27	25	6	4	47	82	A.465	Pus	Aug,2007
37	39	27	25	6	4	47	83	P.16509	Pus	Sept,2007
37	39	27	25	6	4	47	84	P. 16849	Pus	Sept,2007
37	39	27	25	6	4	47	85	P.18339	Pus	Sept,2007
37	39	27	25	6	4	47	86	A.35045	Pus	Feb,2008
46	39	27	25	6	4	48	19	P. 5114	Catheter tip	Oct,2006
36	39	29	25	6	4	49	102	P.1297	Ear Infection	May,2006
37	38	30	25	6	4	50	103	P.5	Sputum	April,2006
37	38	30	24	6	4	51	104	K.6	Sputum	July,2007
37	39	30	24	6	4	52	96	K.11	Pus	Aug,2007
37	39	30	24	6	4	52	97	P.13731	Pus	Aug,2007
37	39	30	25	6	4	53	94	P.4	Pus	April,2006
37	39	30	25	6	4	53	95	P.1287	Pus	May,2006
37	39	27	24	6	4	54	100	P.8668	Ear infection	May,2007
37	39	27	24	6	4	54	101	K.3	Ear infection	July,2007
37	38	27	24	6	4	55	98	P.2886	Catheter tip	Aug,2006
37	38	27	24	6	4	55	99	P.8116	Catheter tip	Dec,2006
34	38	27	24	6	4	56	107	P.1896	Ear infection	June,2006
37	39	21	21	6	4	57	110	A.1761	Ear infection	Sept,2007
45	39	29	24	6	4	58	18	P. 1123	Catheter tip	May,2006
37	38	27	25	5	4	59	87	P.1	Pus	April,2006
37	38	27	25	5	4	59	88	P.12006	Pus	July,2007
37	38	27	25	5	4	59	89	A.9965	Pus	Dec,2007
37	38	27	25	5	4	59	90	A.26163	Pus	Feb,2008
34	38	27	25	5	4	60	106	P.2871	Sputum	Aug,2006
37	29	30	20	6	4	61	112	A.379	Ear infection	Aug,2007
44	50	27	20	7	4	62	118	P.14313	Pus	Aug,2007
44	50	16	20	7	4	63	119	A.13282	Pus	Jan,2008
44	35	25	36	11	4	64	124	NCTC 8325	Standard Strain	
45	35	31	29	10	5	65	125	Mu 50	Standard Strain	

Figure 3. Continued.

Table 2. Spa and MLST types of MRSA strains from Pakistan.

S. aureus isolates	Mec Type	MLVA type	Spa repeat successions/IDs	SPA type	MLST profile aro-arc-glp-gmk-pta-tpi-yqil	MLST type	Clonal complex by RM test
P.2113	IV	MT1	11-12-05-17-34-24-34-22- 25	t451	3-3-1-1-4-62-3	ST113	CC8
P.18431	IV	MT13	11-19-12-05-17-34-24-34-22- 25	t064			CC8
P.8486	IV	MT13	11-19-12-05-17-34-24-34-22- 25	t064			CC8
P.5020	IV	MT13	11-19-12-05-17-34-24-34-22- 25	t064	3-3-1-1-4-62-3	ST113	CC8
P.844	IV	MT13	11-19-12-05-17-34-24-34-22- 25	t064			CC8
A.14254	IV	MT13	11-19-12-05-17-34-24-34-22- 25	t064			CC8
P.17120	IV	MT14	11-19-12-05-17-34-24-34-22- 25	t064			CC8
A.9445	IV	MT17	11-19-12-05-17-34-24-34-22- 25	t064			CC8
A.14256	IV	MT17	11-19-12-05-17-34-24-34-22- 25	t064			CC8
A.15330	IV	MT18	11-19-12-05-17-34-24-34-22- 25	t064	3-3-1-1-4-62-3	ST113	CC8
A.9914	IV	MT18	11-19-12-05-17-34-24-34-22- 25	t064			CC8
K.8	IV	MT18	11-19-12-05-17-34-24-34-22- 25	t064			CC8
P.1863	IV	MT25	11-19-12-05-17-34-24-34-22- 25	t064	3-3-1-1-4-62-3	ST113	CC8
A.9313	III	MT32	15-12-16-02-25-17-24-24	t275	2-3-1-1-4-4-3	ST239	CC8
A.10813	III	MT32	15-12-16-02-25-17-24-24	t275			CC8
A.13282	III	MT63	15-12-16-02-25-17- 24	t037	2-3-1-1-4-4-3	ST239	CC8
P.14947	III	MT27	15-12-16-02-24-16-02-25-17-24	t987			CC8
A.5053	III	MT39	15-12-16-02-24-16-02-25-17-24	t987	2-3-1-1-4-4-3	ST239	CC8
P.1286	IV	MT44	15-12-16-02-16-02-25-17- 24	t021	2-2-2-2-6-3-2	ST30	CC30
P.8	III	MT31	15-12-16-02-24-24	t030			CC8
P.13740	III	MT46	15-12-16-02-24-24	t030			CC8
P.2285	III	MT47	15-12-16-02-24-24	t030	2-3-1-1-4-4-3	ST239	CC8
P.1287	III	MT53	15-12-16-02-24-24	t030			CC8
A.26163	III	MT59	8-16-02-24- 24	t632	2-3-1-1-4-4-3	ST239	CC8
P.12006	III	MT59	8-16-02-24- 24	t632			CC8
P-1	III	MT59	8-16-02-24- 24	t632			CC8

(Enright et al., 2000; McDougal et al., 2003). The prevalence of ST239 is worldwide due its evolution from highly unrelated lineages CC8 and CC30, which resulted in an increased virulence (Edgeworth et al., 2007). The presence of only a limited number of STs in Pakistan predicts local clonal evolution among a relatively conserved society as the major determinant of the distribution of MRSA clones.

We conclude that, although there is significant diversity at the micro level, as shown by variations in the VNTRs, the majority of MRSA strains present in the hospitals of Pakistan are from CC8 and a limited number from CC30. The dominant STs present are ST239 with mec type-III, and ST113 with mec type-IV with only a limited number of strains with an ST30 mec type-IV. Thus, use of MLVA may provide resource poor laboratories with a rapid and robust method for grouping noscomial MRSA isolates into clusters for identification of localized outbreaks. MLVA may also provide an understanding of the evolutionary processes as changes in the number of repeats at different loci may be indicative of which loci are prone to natural selection resulting in higher levels of variation. In this study, we observed more variation in *clfA* and *clfB* than in *sdrC*, *sdrD*, *spa* and *sspa*. We also found that a change in repeat number was not necessarily gradual but may have occurred as a result of large jumps. Some isolates exhibited significant differences in repeat number at a single locus. Further evidence is provided by the spa typing results with a loss of four repeats resulting in a shift from t987 to t030 and a two repeat difference changing t021 to t275 (Table 2). These large jumps might be due to deletions or insertions mediated by recombination or as result of deletions due to slip-strand mispairing during DNA replication. In conclusion, infections by various clones of CC8 especially ST113 and ST239 MRSA are prevalent in Pakistan.

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