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ORIGINAL ARTICLE

Molecular characterization of *Staphylococcus aureus* isolates causing skin and soft tissue infections in patients from Malakand, Pakistan

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Abstract Comparatively few studies have been published describing *Staphylococcus aureus*/MRSA epidemiology in Central Asia including Pakistan. Here, we report the genotyping of *Staphylococcus aureus* strains (that include both methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*) from community- and hospital-acquired skin and soft-tissue infections in a tertiary care hospital in the Malakand district of the Khyber Pakhtunkhwa Province of Pakistan. Forty-five isolates of *Staphylococcus aureus* were characterized by microarray hybridization. Twenty isolates (44 %) were MRSA, whereas 22 (49 %) were PVL-positive. Fourteen isolates (31 %) harboured both *mecA* and PVL genes. The dominant clones were CC121-MSSA ($n = 15$, 33 %) and the PVL-positive "Bengal Bay Clone" (ST772-MRSA-V; $n = 13$, 29 %). The PVL-positive CC8-MRSA-IV strain "USA300" was found once. The pandemic ST239-MRSA-III strain was absent, although it has previously been observed in Pakistan. These observations require a re-assessment of schemes for initial antibiotic therapy to cover MRSA and they emphasise the need for a rapid and non-molecular test for PVL.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major healthcare problem all over the world. MRSA carries *mecA/C* genes that confer resistance to beta-lactams on potentially mobile genetic elements named staphylococcal cassette chromosome *mec*, (SCC*mec*) [1]. Its high genetic variability, the presence of a variety of different strains [2] and a rapid evolution by acquisition of antibiotic resistance and virulence factors complicate infection control and treatment. A large proportion of recently emerging community-acquired MRSA (CA-MRSA) carries phage-borne genes that encode Panton–Valentine leukocidin (PVL) [3]. This is a bicomponent leucocidin [4] that kills leukocytes, or drives them into apoptosis [5]. It is associated with skin and soft-tissue infections and with necrotising pneumonia [6, 7], warranting more stringent infection control and therapy measures than applied for PVL-negative *S. aureus* (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/322857/Guidance_on_the_diagnosis_and_management_of_PVL_associated_SA_infections_in_England_2_Ed.pdf).

Although typing data for *Staphylococcus aureus*/MRSA are abundantly available for Western Europe, the USA or Australia, comparatively few studies have been published describing the situation in the rest of the world, including Central Asia and Pakistan. Three epidemiological studies confirmed the pandemic healthcare-associated strain ST239-MRSA-III to be prevalent in Pakistan. Zafar et al. [8] analysed 126 samples taken in 2006/2007, mainly from Karachi, using pulsed field gel electrophoresis (PFGE), *mecA* PCR, SCC*mec* typing by PCR and multilocus sequence typing (MLST). They found

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ST8-MRSA-IV and ST239-MRSA-III to be the main genotypes in addition to single isolates of ST1 and ST217 (which belongs to CC22).

In a PhD thesis by Arfat [9] on isolates collected in Rawalpindi and Islamabad between 2006 and 2008, hospital-acquired MRSA (HA-MRSA) was analysed by restriction modification (RM), multiple-locus variable-number tandem repeat analysis (MLVA), SCC*mec* typing and MLST. Isolates were assigned to ST239, to ST113 (CC8) and to ST30. In another recent study [10], isolates from Rawalpindi and Islamabad were epidemiologically typed by PFGE, staphylococcal interspersed repeat unit typing (SIRU), RM, SCC*mec* typing, PVL-PCR and MLST. ST239-MRSA-III was found to be the prevalent clone whereas ST8-MRSA-IV, ST113-MRSA-IV and ST30-MRSA-IV [PVL+] were less common.

Another study of skin and soft-tissue infections (SSTI) from Abbottabad in 2009/2010 [11] revealed high rates of MRSA, accounting for 28 % of community- and 48 % of hospital-acquired infections. However, no molecular typing was carried out.

Further studies depict CA-MRSA to disseminate in countries such as Iran [12, 13], Kuwait [14, 15], Oman [16], Saudi Arabia [17] and the Gulf Emirates [2, 18]. The amount of PVL-carrying clones is high in these countries as well. Common MRSA clones are ST6-IV, ST1295-IV and ST772-IV in Oman [16]; ST239-III, CC22-IV, CC30-IV and CC80-IV in Saudi Arabia [17]; and ST22-IV, CC8 and CC80-IV in the Gulf Emirates [2, 18]. Recent typing data also exist for India (with ST2371-IV (CC22), ST22-IV, ST772-V and ST8-IV [19–21]) and Iran (where ST239-III, dominated and ST585, ST2732, ST1294, ST30, ST36 and ST1163 were also found [22]).

Here, we report the genotyping of *S. aureus*, including MSSA, hospital-acquired and community-acquired MRSA, from skin and soft-tissue infections in a (tertiary care) hospital in the Malakand district of the Khyber Pakhtunkhwa Province of Pakistan.

Materials and methods

Sampling area and patient selection

The samples were collected from District Head Quarter Hospital Batkhela Malakand of Khyber Pakhtunkhwa Province between August and September 2015. Study participants were patients in different wards, such as surgical, orthopaedic, dressing room and outpatient departments. Patients having either postsurgical or nosocomial skin and soft-tissue infections or community-acquired skin and soft-tissue infections were selected for sampling. All patients were divided into two broad categories, i.e., those with community-acquired skin and soft-tissue infections (outpatients and patients with infections that manifested within 48 h of admission) and those with healthcare-associated skin and soft-tissue infections (that manifested later than the first 48 h post-admission). All relevant information such as age, gender, occupation, site and type of infection etc. was acquired at the time of sample collection (see also Supplemental Material).

Sampling procedure

The samples were taken using sterile cotton swabs (MWE 170 transwabs; MWE, Corsham, UK). The infection site was first cleansed with alcohol wipes to remove bacteria of the skin's normal flora. The skin lesion content was absorbed by the transwabs and inserted and sealed in the tube with transport medium. The samples were transported to the microbiology laboratory of the University of Haripur for culturing.

Only one sample per patient was included. If it yielded phenotypically different colonies, both variants were tested separately. They were analysed separately only when genotypically different.

Identification of *S. aureus*

Mannitol salt agar (MSA; Oxoid, Basingstoke, UK) was used as a selective medium for growing *S. aureus*. Transwabs with patient samples were streaked on MSA plates and incubated at 37 °C for 24–48 h. *S. aureus* strains were confirmed by using conventional microbiological tests, i.e. microscopy, catalase and coagulase assays and mannitol fermentation. The *S. aureus* strains identified were stored in brain heart infusion broth with 20 % glycerol at −20 °C.

Array procedures

The characterization of isolates was performed using StaphyType DNA microarrays (Alere Technologies, Jena, Germany), which cover 333 different target sequences corresponding to approximately 170 distinct genes and their allelic variants. These genes include species markers, typing markers, in addition to toxin genes and resistance genes. Detailed descriptions of genes, sequences and protocols have been published previously [2, 23]. In brief: sample swabs were inoculated on Columbia blood agar. *S. aureus* was sub-cloned on a Columbia blood agar plate, harvested and enzymatically lysed. DNA was purified using Qiagen spin columns. A linear amplification was then performed with one specific primer for each target. This method allows simultaneous amplification of a multitude of target sequences, but compared with PCR it results in a lower number of copies owing to its linear kinetics. Biotin-dUTP was incorporated into the amplicons during amplification, thus allowing the detection of hybridization to probes immobilized to the array. Detection was performed

using streptavidin–horseradish peroxidase, which catalysed a local precipitation of a dye. This resulted in visible spots on the microarray that were imaged and analysed using a designated reader and software (Alere Technologies). The patterns of spots allowed the presence or absence of certain genes or alleles to be established, in addition to, by automated comparison with a database, assignment to clonal complexes, strains and SCC*mec* types.

Results

In this study, 45 isolates of *S. aureus* from skin and soft-tissue infections were characterized. They were assigned to seven clonal complexes and ten distinct strains. Forty-four percent of isolates were MRSA, 49 % were PVL-positive and 31 % harboured both *mecA* and PVL genes. Tables 1 and 2 show the prevalence data for genes associated with resistance and virulence. Full data are shown in the Supplemental Material.

Table 1 Presence of resistance genes in skin and soft-tissue infections (SSTI) isolates from Malakand

Group	Gene	*n*	%
Genes associated with beta-lactam resistance	*mecA*	20	44.44
	mecC	0	0.00
	blaZ	39	86.67
SCC*mec* types	SCC*mec* I	0	0.00
	SCC*mec* II	0	0.00
	SCC*mec* III	0	0.00
	SCC*mec* IV	7	15.56
	SCC*mec* V	13	28.89
	SCC*mec* VI to XII	0	0.00
Genes associated with macrolide/lincosamide resistance	*ermA*	0	0.00
	ermB	0	0.00
	ermC	0	0.00
	linA	1	2.22
	mpbBM	19	42.22
	msrA	19	42.22
Genes associated with amino glycoside resistance	*aacA-aphD*	13	28.89
	aadD	1	2.22
	aphA3	20	44.44
Genes associated with fusidic acid resistance	*far1/fusB*	0	0.00
	fusC	0	0.00
Miscellaneous resistance genes	*cat*	0	0.00
	cfr	0	0.00
	dfrA	0	0.00
	mupR	0	0.00
	sat	20	44.44
	tetK	3	6.67
	tetM	0	0.00
	vanA	0	0.00

Table 2 Presence of virulence factors in SSTI isolates from Malakand

Group	Gene(s)	*n*	%
agr group (alleles of accessory gene regulator locus)	*agr* I	8	17.78
	agr II	13	28.89
	agr III	8	17.78
	agr IV	16	35.56
Toxic shock syndrome toxin gene	*tst1*	0	0.00
Enterotoxin genes	*sea*	20	44.44
	sea-N315 = *sep*	0	0.00
	seb + *sek* + *seq*	1	2.22
	sec + *sel*	14	31.11
	sed, sej, ser	0	0.00
	see	0	0.00
	egc gene cluster	37	82.22
	ORF CM14	30	66.67
Panton–Valentine leukocidin	*lukF/S*-PV	22	48.89
Genes associated with haemolysin beta integrating phages	*sak*	32	71.11
	chp	11	24.44
	scn	44	97.78
Exfoliative toxin genes	*etA*	0	0.00
	etB	15	33.33
	etD	2	4.44
Miscellaneous virulence genes	*edinA*	0	0.00
	edinB	2	4.44
	edinC	14	31.11
	ACME	1	2.22
Capsule types	Capsule type 5	16	35.56
	Capsule type 8	29	64.44
Adhesins	*cna*	42	93.33
	sasG	20	44.44

Assignment to strains and clonal complexes was carried out on the basis of a hybridization pattern, as described previously [2, 23]. Detailed strain descriptions are listed below, and an overview of the distribution of clones and relevant genes is given in Table 3. The dominant clones were CC121-MSSA (33 %) and the PVL-positive "Bengal Bay clone" (ST772-MRSA-V; 29 %). The remaining third of the isolates comprised another eight different strains. ST239 was completely absent.

CC1/ST772-MRSA-V (Bengal Bay clone)

ST772 is usually assigned to CC1 based on the identity of six out of seven MLST genes, but it differs in several key markers such as capsule type (5 in ST772, but 8 in ST1), *agr* group affiliation (II for ST772, but III for ST1), absence of the enterotoxin gene *seh* or the presence of the *egc* enterotoxin gene

Table 3 Strain assignments of SSTI isolates from Malakand

Clonal complex	Strain	*n*	%	CA	HA
1 (ST772)	ST772-MRSA-V [PVL+], "Bengal Bay clone"	13	28.89	11	2
6	CC6-MRSA-IV, "WA MRSA-51"	5	11.11	3	1
8	CC8-MRSA-IV [PVL+/ACME+], "USA300"	1	2.22	1	0
30	CC30-MSSA	1	2.22	0	1
	CC30-MSSA [PVL+]	6	13.33	5	1
121	CC121-MSSA	15	33.33	9	6
	CC121-MSSA [PVL+]	1	2.22	1	0
398 (ST291/813)	ST291/813-MSSA	1	2.22	1	0
	ST291/813-MSSA [PVL+]	1	2.22	0	1
509	CC509-MRSA-IV	1	2.22	1	0

CA community-acquired, *HA* healthcare-acquired

cluster [24, 25]. Thirteen identical isolates were assigned to this lineage, and to the ST772-MRSA-V strain known as "Bengal Bay clone" [26], all being positive for PVL genes and for SCC*mec* V. They also harboured enterotoxin genes *sea*, *sec* and *sel*, the gene *scn* (staphylococcal complement inhibitor) and, for a community-acquired strain, an unusually high number of antibiotic resistance genes, i.e. *blaZ* (beta-lactamase), *msr*(A), *mph*(C) (macrolide resistance), *aacA-aphD* (gentamicin and tobramycin resistance), *aphA3* (kana-/neomycin resistance) and *sat* (streptothricin resistance). Two out of these 13 isolates were recorded as being hospital-acquired.

CC6-MRSA-IV (WA MRSA-51)

Five isolates were identified as CC6-MRSA-IV, a strain first described in Australia as WA MRSA-51 [27]. All isolates carried *sea*, but lacked PVL genes, and they did not harbour additional resistance genes beside *mecA*.

ST8-MRSA-IV [ACME/PVL+] (USA300)

One isolate was assigned to USA300, i.e. PVL- and ACME-positive CC8-MRSA-IV. It appeared to be identical to the fully sequenced strain TCH1516, GenBank CP000730.1.

CC30-MSSA

Seven isolates belonged to CC30-MSSA, 6 of them were PVL-positive. Five of the PVL-positives carried the antibiotic resistance genes *blaZ*, *msr*(A), *mph*(C), *aphA3* and *sat*. A sixth one harboured *blaZ*, *linA* (clindamycin/lincosamide resistance), *aadD* (tobramycin resistance), *tetK* (tetracycline resistance) in addition to the enterotoxin A gene *sea*. The PVL-negative isolate lacked all the resistance markers, but also carried *sea*.

CC121-MSSA

Sixteen isolates belonged to CC121. A single one was PVL-positive, and no CC121-MRSA was detected. All CC121 isolates carried the *egc* locus and the enterotoxin homologue ORF CM14. The PVL-positive isolate additionally harboured the enterotoxin B gene *seb*. Fourteen out of 15 PVL-negative isolates carried the genes for exfoliative toxin (*etB*) and epidermal cell differentiation inhibitor C (*edinC*). The 15th isolate proved to be a mixed culture of one *etB*- and *edinC*-positive variant and a second variant that was negative for these two genes. No resistance genes besides *blaZ* were detected. A higher rate of hospital-acquired cases was observed for the PVL-negative CC121-MSSA strain than for other strains from this study (Table 3), suggesting a possible in-house transmission.

ST291/813-MSSA

Two isolates showed the characteristic hybridization profile of ST291/813. Although this lineage is—according to MLST—considered a double locus variant of ST398, genome sequencing data indicate that it is separate entity [28, 29]. As CC398, these isolates are *agr* group I and capsule type 5. However, they do not yield signals for *coa* and *cna*, and they differ from ST398 in the alleles of *ssl01*, *ebpS*, *sdrD* and *hsdS*, and in the presence of leucocidin *lukE* and *spl* protease genes. Both isolates harboured *blaZ*, and genes encoding an exfoliative toxin, *etD*, and an epidermal cell differentiation inhibitor, *edinB*; one was PVL-positive.

CC509-MRSA-IV

One isolate of CC509-MRSA-IV was found. It belonged to *agr* group III and capsule type 8. It carries resistance genes *mecA*, *blaZ*, *aphA3*, *sat* and *tetK*. Enterotoxin gene cluster genes *selm* and *selo* were detected; other probes for *egc* genes did not yield signals. Genes ORF CM14 in addition to *etB* and *edinC* were present.

Discussion

With regard to MRSA epidemiology, different clones were detected from those in previous studies from Pakistan [8–10]. There, ST239-MRSA-III and ST8-MRSA-IV, in addition to other, less common clones were found.

In our study, we observed a predominance of a PVL-positive ST772-MRSA-V strain. This clone was previously dubbed "Bengal Bay clone" because its first cases were associated with origin from or travel to regions around the Bay of Bengal [26] where this strain became endemic [25, 30, 31], displacing previous predominant HA-MRSA clones in a hospital setting, but also spreading in the community [20]. A local spread has also been reported in Oman [16]. Imported cases were observed in many countries including UK, Ireland, Italy, Norway, Abu Dhabi, the Kingdom of Saudi Arabia, India, New Zealand, Australia, Hong Kong and others [2, 17, 24, 32–35], mostly associated with travel into endemic regions. The distribution of this clone may illustrate an epidemiological link between Pakistan and the Arabian Peninsula, where approximately 2,000,000 Pakistani citizens are employed [36].

Another MRSA strain that was found is CC6-MRSA-IV. This strain was apparently first observed in Australia [27], but also appears to be a common strain in the Middle East. It was found in the Kingdom of Saudi Arabia [17], Kuwait [37], Oman [16] and Abu Dhabi [2, 18] so that a dissemination of epidemic strains in either direction appears to be possible. Interestingly, CC6-MSSA was found to be a common clone in camels in Dubai, thus suggesting a zoonotic transmission from camels to humans and acquisition of a SCC*mec* element [38].

CC509-MRSA-IV was only once observed in Western Australia, but this isolate originated from a patient without any epidemiological link to Central Asia/Pakistan and differed in carriage of accessory resistance genes (Geoffrey Coombs/Julie Pearson, Perth, WA, Australia; personal communication). Thus, no assumptions can currently be made on the origin and provenance of this strain.

A further sporadic MRSA clone was USA300. This strain is known to be widespread in the USA, Australia and Western Europe [2, 39–43], but for Central Asia, there are no data available yet. Again, a link to the Gulf may be possible, as this strain was detected in Abu Dhabi [2, 18].

However, other MRSA clones that have been reported from the Middle East, such as CC22 or CC80 MRSA, were not found in this study. Most conspicuously, the pandemic ST239-MRSA-III strain was not identified, although earlier studies described it to be common in Pakistan. To confirm its possible demise or disappearance, however, more isolates should be typed and emphasis should be placed on other typical healthcare-associated infections (such as ventilator-associated pneumonia).

With this caveat, our observation may indicate a shift in the population structure of MRSA within the last decade. Although studies from samples taken in 2006 and 2010 revealed ST239, ST8 and ST113 to be the prevalent clones [8–10], these clones are now absent, being replaced largely by the emerging PVL-positive ST772-MRSA-V strain. The ST239 strain used to be a pandemic clone, but it was strictly confined to hospital settings and apparently never spread in the community outside of healthcare facilities. With the emergence of the virulent and rather multi-resistant ST772-MRSA-V strain, MRSA is no longer confined to hospitals, and the abundance of this strain outside of hospitals will make infection control even more difficult. This trend is also observed in other regions [20, 32].

Regarding MSSA, virtually no typing data are available for Pakistan. CC30 and CC121 are both pandemic clones that can essentially be found everywhere [44–51]. A third lineage, ST291/813, was already reported from Pakistan [29], but also from Iran [52], India [53], Mali [54], Romania [51], Algeria and Western Europe [28, 46, 55, 56]. It was also found in dairy milk of Egyptian cows and buffalo [57] and this may indicate a zoonotic origin of this lineage.

The overall results of this study are observations of a high rate of MRSA and a high rate of PVL-positive isolates. There was a high prevalence of both in cases that were classified as community-associated (Table 3). In the wake of these observations a re-assessment of antibiotic therapy schemes are warranted that locally rely on amoxicillin + clavulanic acid, cefixime and ceftriaxone. For instance all patients with ST772-MRSA-V received these beta-lactams (see Supplemental Material). Our observations also emphasize a need for a rapid and non-molecular test for PVL, as described previously [48], and suggest a combined PBP2a + PVL lateral flow assay systems for use in regions with a high burden of MRSA and of PVL-positives, such as Pakistan.

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Compliance with ethical standards

Conflicts of interest Sara Madzgalla, Elke Müller, Annett Reissig, Ralf Ehricht and Stefan Monecke are employees of Alere Technologies, Jena, Germany, the company that manufactures the microarrays used for this study.

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Informed consent and ethical approval Informed written consent was taken from each patient and the study was approved by the institutional ethics committee.

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