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ORIGINAL ARTICLE

Hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) from Pakistan: molecular characterisation by microarray technology

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Abstract

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in Pakistan is known to be high, but very few studies have described the molecular epidemiology of the different MRSA clones circulating in the country. Forty-four MRSA isolates were collected from two tertiary care hospitals of the Rawalpindi district of Pakistan. All strains were identified by a conventional phenotypic method and then subjected to genotyping by microarray hybridisation. Six clonal complexes (CCs) and 19 strains were identified. The most commonly identified strains were: (i) Panton–Valentine leucocidin (PVL)-positive CC772-MRSA-V, "Bengal Bay Clone" (ten isolates; 22.3%), (ii) ST239-MRSA [III + *ccrC*] (five isolates) and (iii) a CC8-MRSA-IV strain, as well as CC6-MRSA-IV (both with four isolates; 9.1% each). Several of the strains detected indicated epidemiological links to the Middle Eastern/Arabian Gulf region. Further studies are needed to type MRSA from countries with less known epidemiology and to monitor the distribution and spread of strains, as well as possible links to global travel, migration and commerce.

Introduction

Methicillin resistance in staphylococci is associated with *mec* genes (*mecA*, *mecC*) located on a large transmissible element known as staphylococcal cassette chromosome *mec* (SCC*mec*) [1–5]. The first strains of methicillin-resistant *Staphylococcus aureus* (MRSA) appeared shortly after the introduction of penicillinase-resistant penicillins into clinical practice, in 1961 [6]. MRSA was considered to be a nosocomial pathogen causing serious infections in individuals with healthcare-associated risk factors [7]. However, more recent reports of de novo community-associated MRSA (CA-MRSA) infections among healthy individuals began to appear in the literature in the 1990s. They were soon shown to be associated with genetically distinct, and diverse, lineages of MRSA, apparently unrelated to existing healthcare-associated MRSA (HA-MRSA) strains [8, 9], as shown by molecular typing methods such as multilocus sequence typing (MLST, [10]) that differentiates sequence types (ST) and clusters them to lineages named clonal complexes (CC). Soon thereafter,

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numerous lineages of CA-MRSA emerged on every continent [11, 12]. CA-MRSA strains are increasingly involved in nosocomial infections [13] being brought into hospital with admissions from an affected community. Consequently, traditional distinctions between HA-MRSA and CA-MRSA based on molecular typing and susceptibility profiles are becoming increasingly less relevant [14], and this distinction should be made based on individual case histories only.

Meanwhile, 12 different main SCC*mec* types and a wide variety of variants [15] have been described. The traditional HA-MRSA clones are characterised by possession of types I, II and III, with type I strains slowly disappearing, while strains with SCC*mec* type II (CC5-MRSA-II, "New York/Japan Clone") and SCC*mec* type III (ST239-MRSA-III, "Vienna/Hungarian/Brazilian Clone") can still be considered pandemic. CA-MRSA strains typically feature smaller type IV and V elements (see [1–5]; http://www.sccmec.org/Pages/SCC_TypesEN.html). Currently, worldwide reports of CA-MRSA are associated with > 20 distinct genetic lineages, or CCs, five of which are globally predominant, including CC1-MRSA-IV (including strains "WA-1" and "USA400"), CC8-MRSA-IV (including strains "USA300", "Lyon Clone" and others), ST30-MRSA-IV ("SWP clone"), ST59-MRSA-V ("Taiwan Clone") and ST80-MRSA-IV ("European"/Middle Eastern clone). While there is a multitude of data on population structure and changes in that structure for Western Europe, Japan, Australia and the USA, such data are lacking for many other parts of the world. This, unfortunately, includes Pakistan. It is known that the MRSA prevalence is high as many local studies from Pakistan indicate. A prevalence of 22.9% among clinical *S. aureus* isolates was reported by Akhter et al. [16], of 27% by Ajmal et al. [17] and of ranges of as much as 42 to 51% by Butt et al. [18], Hafiz et al. [19], Perwaiz et al. [20] and Hussain et al. [21]. Nonetheless, all these studies did not apply molecular typing tools. Thus, there are very limited epidemiological typing data available from this region [22]. Zafar et al. [23] carried out a study in 2006–2007 to investigate the prevalent genotypes and isolates were characterised by pulsed-field gel electrophoresis (PFGE), SCC*mec* typing and MLST. The data indicated ST8 and ST239 clones to be the most prevalent. Another study conducted by Shabir et al. [22] also showed ST239-MRSA-III as the dominant strain. A very recent study conducted by Madzgalla et al. [24] indicated the "Bengal Bay Clone" (ST772-MRSA-V) to be the most prevalent clone. This strain has emerged as a virulent and unusually resistant CA-MRSA strain in Bangladesh and India, and is increasingly reported in the Middle East, UK and Europe [25–35]. However, this study focused on skin and soft tissue infections, many of which were community-acquired and/or caused by methicillin-susceptible strains. In order to gather data on hospital-acquired MRSA from Pakistan, 44 MRSA clinical isolates were collected for the present study from two tertiary care hospitals and subjected to microarray technology to type and characterise them.

Materials and methods

Isolate collection

The study comprised of 44 MRSA isolates that have been collected consecutively from January to March 2017 at a laboratory serving the renal units of two tertiary care hospitals in Rawalpindi, Al-Sayed Hospital (ASH) and Fauji Foundation Hospital (FFH). Isolates originated from routine clinical specimens, which included pus (30 isolates), nasal swabs (nine isolates), central venous catheters (two isolates), bronchoalveolar lavage (one isolate), blood culture (one isolate) and thoracic swab (one isolate); for more details, see the supplemental material.

Each specimen was inoculated on blood agar plates prepared from commercially available dehydrated media (Oxoid, Basingstoke, UK).

Inoculated plates were incubated aerobically for 24 h at 37 °C and then examined for growth. Isolates suspected to be staphylococci were further inoculated on Mannitol salt agar (MSA; Oxoid, Basingstoke, UK) as a selective medium for growing *S. aureus*. Only those strains that showed mannitol fermentation indicated by yellow colouring of the medium surrounding colonies were selected. *S. aureus* was confirmed by using conventional microbiological tests, i.e. microscopy, catalase and coagulase assays. The pure cultures were stored in glycerol stocks at − 80 °C.

Susceptibility tests

Antimicrobial susceptibility testing was performed according to the modified Kirby–Bauer disc diffusion technique on Mueller–Hinton agar (Oxoid, Basingstoke, UK). Commercially available antimicrobial discs for penicillin (10 μg), ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), cefoxitin (30 μg), erythromycin (15 μg), clindamycin (2 μg), gentamicin (10 μg), co-trimoxazole (1.25/23.75 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), doxycycline (30 μg), levofloxacin (5 μg), amikacin (30 μg), fusidic acid (10 μg), vancomycin (30 μg), linezolid (30 μg), teicoplanin (30 μg) and tigecycline (15 μg) were employed for this purpose and zones were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) standards. The isolates were considered methicillin resistant if the zone of inhibition was ≤ 21 mm.

Twenty-two isolates were re-tested by VITEK-2 with AST P608 cards according to the manufacturer's instructions

(bioMérieux, Nürtingen, Germany). These included two clones obtained from one mixed culture, isolates for which phenotypic and genotypic data were not in full concordance and isolates in which contaminations of transport media were observed or suspected after shipment.

The data are summarised in the supplemental material.

Microarrays

The molecular characterisations of these MRSA isolates were performed with Alere StaphyType DNA microarrays Abbott (Alere Technologies GmbH), Jena, Germany; cat. nr. 245200096). The array contains 333 target sequences that correspond to approximately 170 distinct genes, such as species markers, typing markers for SCC*mec* (staphylococcal cassette chromosome), capsule types and *agr* groups, as well as resistance genes and genes encoding exotoxins. All primers, probes, amplification as well as hybridisation protocols were as previously published [11, 36]. The assignment to CCs was performed using the hybridisation patterns onto microarrays, as previously described [11, 36].

For further characterisation of SCC*mec* elements, all isolates were analysed with another microarray (*S. aureus* Genotyping Kit 2.1 SCC; Abbott (Alere Technologies GmbH), Jena, Germany; cat. nr. 247200096). The relevant target genes, probes and primers were previously disclosed by Monecke et al. [15]. The procedures for all microarray experiments were carried out as described above.

Results

Resistance and virulence genes

Tables 1 and 2 show the prevalence data for the most frequently detected resistance and virulence genes. Full datasets as well as the susceptibility test results are provided in the supplemental material. Genes that were not found are not shown in Tables 1 and 2 but they are listed in the supplemental material.

All isolates were *mecA* positive and resistant to all tested beta-lactams. MRSA with *mecC* were not identified. Resistance to fluoroquinolones, aminoglycosides, tetracyclines and co-trimoxazole was common (see the supplemental material). Vancomycin or linezolid resistance genes (*vanA* and *cfr*) were not detected, and no glycopeptide or linezolid resistance was observed. The mupirocin resistance gene (*mupA*) was found in two isolates.

Regarding virulence factors, 15 isolates (34.09%) were Panton–Valentine leucocidin (PVL)-positive, of which 13 belonged to CC772, one to the pandemic "USA300" strain and one to another CC8 MRSA strain (see below).

Table 1 Detection and prevalence rates of selected resistance genes. Genes that were not found during the study are not shown. They are listed in the supplemental material

Group	Gene designation	Number of positive isolates	Percentage (%)
Genes for beta-lactam resistance	*mecA*	44	100.00
	blaZ	43	97.73
SCC*mec* main types	SCC*mec* II	1	2.27
	SCC*mec* III	6	13.63
	SCC*mec* IV	23	52.27
	SCC*mec* V	11	25.00
	SCC*mec* VT	3	6.81
Genes for macrolide/lincosamide resistance	*ermA*	6	13.64
	ermC	16	36.36
	linA	2	4.55
	mpbBM (= *mph*(*C*))	17	38.64
	msrA	17	38.64
Genes for aminoglycoside resistance	*aacA-aphD*	28	63.64
	aadD	7	15.91
	ble	5	11.36
	aadE	24	54.55
	aphA3	24	54.55
Genes for fusidic acid resistance	*far1*/*fusB*	1	2.27
	fusC	1	2.27
Miscellaneous resistance genes	*cat*	2	4.55
	dfrA	4	9.09
	dfrG	27	61.36
	mupA	2	4.54
	sat	24	54.55
	tetK	6	13.64
	tetM	12	27.27
Heavy metal resistance genes	*merA*/*B*	1	2.27
	Plasmidic *mco*	5	11.36
	Plasmidic *copA2*	5	11.36
	copA2-SCC	2	4.55
	arsB-SCC	2	4.55
	arsC-SCC	1	2.27
	czrC	2	4.55
	cadD (*R35*)	7	15.91

Molecular typing and strain affiliations

In this study, six different CCs and 19 different strains (as defined by CC affiliation, toxin gene carriage and SCC*mec* subtype) were identified. Detailed strain descriptions and an overview of the typed strains and relevant target genes are listed in Table 3. Details on SCC*mec* elements are given in Table 4.

Table 2 Detection and prevalence rates of selected virulence-associated genes. Genes that were not found during the study are not shown. They are listed in the supplemental material

Group	Gene designation	Number of positive isolates	Percentage (%)
agr group (alleles of accessory gene regulator locus)	*agr* I	27	61.36
	agr II	17	38.64
Toxic shock syndrome toxin gene	*tst1*	6	13.64
Enterotoxins	*sea*	23	52.27
	seb	4	9.09
	sec	18	40.91
	sek	11	25.00
	sel	18	40.90
	seq	11	25.00
	egc gene cluster (*seg* + *i* + *m* + *n* + *o* + *u*)	22	50.00
	ORF CM14	14	31.82
Panton–Valentine leucocidin (PVL)	*lukF* + *S*-PV	15	34.09
Genes associated with haemolysin beta integrating phages	*sak*	28	63.64
	chp	8	18.18
	scn	42	95.45
Miscellaneous virulence genes	*edinA*	2	4.55
	tirS	1	2.27
	ACME	1	2.27
Capsule types	Capsule type 5	34	77.27
	Capsule type 8	10	22.73
Adhaesins	*cna*	29	65.91
	sasX	1	2.27
	sasG	43	97.73

The most commonly identified strains were: (i) PVL-positive CC772-MRSA-V, "Bengal Bay Clone" (ten isolates; 22.3%), (ii) ST239-MRSA [III + *ccrC*] (five isolates) and (iii) a CC8-MRSA-IV strain, as well as CC6-MRSA-IV (both with four isolates; 9.1% each).

In the following paragraphs, CCs and strains will be discussed.

CC5-MRSA

Two different CC5-MRSA strains were identified. One carried SCC*mec* IVa, thus resembling the internationally known "Paediatric clone", but was positive for *tst1* and *edinA*. The other one had a composite SCC*mec* IV/SCC*fus* element, being similar or identical to a strain previously described from Malta [37] and the Middle East [38].

CC6-MRSA-IV

CC6-MRSA-IV was detected four times. This strain was previously observed in Western Australia (hence, "WA MRSA-51"; [39]), but also in the Middle East [34, 38] and Pakistan [24]. All isolates contained SCC*mec* IV elements, and one was subtyped yielding an SCC*mec* IVa element as in MW2 (GenBank BA000033.2, see Table 4). They did not carry the PVL genes and no additional resistance genes besides *mecA* and *blaZ*.

CC8-MRSA

Twelve isolates were assigned to CC8; ST239 is to be discussed separately (see below).

One isolate resembled the Irish AR13 strain in having an SCC*mec* II element that lacked the *kdp* operon. However, it differed in the presence of cadmium and arsenic resistance genes (Table 4).

Two further isolates were PVL-positive and also positive for enterotoxin genes K and Q. One had an SCC*mec* IV + ACME1 composite element as known from the pandemic "USA300" strain. The other one had a plain (MW2-like) SCC*mec* IVa, thus resembling the genome sequence of IS-88, GenBank AHLO.

Furthermore, four PVL-negative and rather multi-resistant (see the supplemental material) CC8-MRSA-IV strains were observed that differed in SCC*mec* subtype (SCC*mec* IVa or IVc), as well as in the presence, or absence, of enterotoxin genes *seb*, *sek* and *seq*.

Table 3 Clonal complex (CC) assignments, numbers, prevalence rates and descriptions of the methicillin-resistant *Staphylococcus aureus* (MRSA) strains detected during this study

CC	Strain	No.	%	*agr*/ capsule type	SCC*mec* type* (number subtyped)	Other resistance genes	PVL	*tst1*	*egc*	Other virulence factors	Adhesion factors
CC5	CC5-MRSA-IV (*tst1*+), "Paediatric clone"	2	4.55	*agr* II/cap5	SCC*mec* IVa $_{(MW2)}$	*blaZ*, *ermC* (1 out of 2), *msrA*/*mpbBM* (1/2), *dfrG* (1/2), *aadE*/*aphA3*/*sat* (1/2)	–	POS	POS	*sec*/*l*, *lukD*/*E*, *edinA*, *sak*, *chp*, *scn*	*sasG*
	CC5-MRSA-[IV + *fus* + *ccrAB*], "Maltese Clone"	1	2.27	*agr* II/cap 5	SCC [*mec* IVa + *fus* + *tir* + *ccrA-B*] $_{(CMFT181)}$	*fusC*, *blaZ*	–	POS	POS	*sea*, *sec*/*l*, *lukD*/*E*, *sak*, *scn*	*sasG*
CC6	CC6-MRSA-IV, "WA MRSA-51"	4	9.09	*agr* I/cap 8	SCC*mec* IVa $_{(MW2)}$	*blaZ*	–	–	–	*sea*, *lukD*/*E*, *sak*, *scn*	*cna*, *sasG*
CC8	ST8-MRSA-IIA/B/D, similar to "Irish AR13/14"	1	2.27	*agr* I/cap 5	SCC [*mec* II (kdp-) + As/Cd)] (Unknown)	*arsB*, *cadD* $_{(R35)}$, *blaZ*, *ermC*, *linA*, *msrA*/*mpbBM*, *dfrG*, *aacA-aphD*, *aadE*/*aphA3*/*sat*, *tetM*, *qacA*	–	–	–	*lukD*/*E*, *sak*, *scn*	*sasG*
	CC8-MRSA-IVa (*seb*/*k*/*q* −)	4	9.09	*agr* I/cap 5	SCC*mec* IVa $_{(MW2)}$	*cadX*, *blaZ*, *ermC*, *aacA-aphD*, *aadD* (2/4), *dfrG*, *aadE*/*aphA3*/*sat*, *tetM*, *mupA* (2/4), *qacA*, *qacC* (2/4)	–	–	–	*lukD*/*E*, *sak*, *scn*	*sasG*
	CC8-MRSA-IVa (*seb*/*k*/*q* +)	1	2.27	*agr* I/cap 5	SCC*mec* IVa $_{(MW2)}$	*cadX*, *blaZ*, *ermC*, *dfrG*, *aacA-aphD*, *aadE*/*aphA3*/*sat*, *tetM*	–	–	–	*seb*/*k*/*q*, *lukD*/*E*, *sak*, *scn*	*sasG*
	CC8-MRSA-IVc (*seb*/*k*/*q* −)	1	2.27	*agr* I/cap 5	SCC*mec* IVc $_{(IS-105)}$	*blaZ*, *ermC*, *dfrG*, *aacA-aphD*, *aadD*, *ble*, *tetK*, *qacC*	–	–	–	*lukD*/*E*, *sak*, *scn*	*sasG*
	CC8-MRSA-IVc (*seb*/*k*/*q* +)	3	6.82	*agr* I/cap 5	SCC*mec* IVc $_{(IS-105)}$	*cadX* (1/3), *blaZ*, *ermC* (1/3), *dfrG*, *aacA-aphD*, *aadD*, *ble*, *aadE*/*aphA3*/*sat* (1/3), *cat* (2/3)	–	–	–	*seb*/*k*/*q*, *lukD*/*E*, *sak*, *scn*	*sasG*
	ST8-MRSA-[IV + ACME] (PVL+), "USA300"	1	2.27	*agr* I/cap 5	SCC [*mec* IVa + ACME1 + Cu] (USA300)	*copA2*	POS	–	–	*sek*/*q*, *lukD*/*E*, *sak*, *chp*, *scn*, ACME	*sasG*
	CC8-MRSA-IV (PVL+)	1	2.27	*agr* I/cap 5	SCC*mec* IVa $_{(MW2)}$	*blaZ*, *msrA*/*mpbBM*, *aadE*/*aphA3*/*sat*	POS	–	–	*sek*/*q*, *lukD*/*E*, *sak*, *chp*, *scn*	*sasG*
CC22	CC22-MRSA-IV (*fnbB*+), similar to "EMRSA-15"	1	2.27	*agr* I/cap 5	SCC*mec* IVa $_{(MW2)}$	*blaZ*, *ermC*, *tetK*	–	–	POS	*sak*, *chp*, *scn*	*cna*, *sasG*
	CC22-MRSA-IV (*tst1*+)	3	6.82	*agr* I/cap 5	SCC*mec* IVa $_{(CMFT503)}$	*blaZ*, *ermC* (1/3), *dfrA*, *tetK* (2/3)	–	POS	POS	*sak*, *chp*, *scn* (2/3)	*cna*, *sasG*
	CC22-MRSA-VT	1	2.27	*agr* I/cap 5	SCC*mec* VT $_{(H121820035)}$	*blaZ*, *ermC*, *dfrG*	–	–	POS	*sec*/*l*, *sak*, *chp*, *scn*	*cna*, *sasG*
CC239	ST239-MRSA-[III + *ccrC*]	5	11.36	*agr* I/cap 8	SCC [*mec* III + Cd + *ccrC*] $_{(CN79)}$	*cadD* $_{(R35)}$, *blaZ*, *ermA*, *ermC* (4/5), *aacA-aphD*, *tetM*	–	–	–	*sea*, *sek*/*q*, *lukD*/*E*, *sak*, *scn*	*cna*, *sasG* (4/5)
	ST239-MRSA-[III + Hg + *ccrC*]	1	2.27	*agr* I/cap 8	SCC [*mec* III + Cd/Hg + *ccrC*] (Bmb9393)	*cadD* $_{(R35)}$, *merA*/*B*, *blaZ*, *ermA*, *dfrG*, *aacA-aphD*, *aadE*/*aphA3*/*sat*, *tetM*	–	–	–	*lukD*/*E*	*cna*, *sasG*, *sasX*
CC772	CC772-MRSA-[irreg. SCC*mec*] (PVL+)	1	2.27	*agr* II/cap 5	SCC [*mec* IV + As/Cu + *ccrC*] (Unknown)	*copA2*, *arsB*/*C*, *blaZ*, *msrA*/*mpbBM*, *dfrG*, *aacA-aphD*, *ble*, *aadD*, *aadE*/*aphA3*/*sat*, *dfrA*, *far1*	POS	–	POS	*sea*, *sec*/*l*, ORF CM14, *scn*	*cna*, *sasG*
	CC772-MRSA-V	1	2.27	*agr* II/cap 5	SCC*mec* V $_{(Bengal\ Bay)}$	*blaZ*, *msrA*/*mpbBM*, *dfrG*, *aacA-aphD*, *aadE*/*aphA3*/*sat*	–	–	POS	*sec*/*l*, ORF CM14, *scn*	*cna*, *sasG*
	CC772-MRSA-V (PVL+), "Bengal Bay Clone"	10	22.72	*agr* II/cap 5	SCC*mec* V $_{(Bengal\ Bay)}$	*blaZ*, *msrA*/*mpbBM*, *linA* (1/10), *dfrG*, *aacA-aphD*, *aadE*/*aphA3*/*sat*	POS	–	POS	*sea*, *sec*/*l*, ORF CM14, *scn*	*cna*, *sasG*
	CC772-MRSA-VT (PVL+)	2	4.55	*agr* II/cap 5	SCC [*mec* VT + *czrC*] (SO385)	*blaZ*, *msrA*/*mpbBM*, *dfrG*, *aadE*/*aphA3*/*sat*, *tetK*	POS	–	POS	*sea*, *sec*/*l*, ORF CM14, *scn*	*cna*, *sasG*

*Subscripted strain names in SCC*mec* designations indicate reference strains with identical SCC-related patterns (see also Table 4)

CC22-MRSA

A total of six isolates were identified and assigned to three different strains. One strain (four isolates) was a *tst1*-positive CC22-MRSA-IV yielding a hybridisation pattern of SCC*mec* markers consistent with an SCC*mec* IV element as sequenced in CMFT503, GenBank HF569113.1. Another strain was a *tst1*-negative CC22-MRSA-IV that closely resembled the pandemic Barnim/UK-15 MRSA strain. It differed, however, in SCC*mec* subtype (SCC*mec* IVa as in MW2) and in signals for *fnbB* probes (for the issue of *fnbB* in CC22-MRSA, see [40]). Finally, one additional isolate harboured an SCC*mec* VT element.

ST239-MRSA ("Vienna/Hungarian/Brazilian clone")

Based on the MLST data, ST239 has been considered as part of CC8 but it is to be discussed here separately because of many genotypic differences caused by the integration of a large fragment of CC30 DNA [41].

In the present study, a total of six isolates was assigned to ST239. These harboured SCC*mec* III as well as an additional recombinase gene (*ccrC*). One isolate differed in a presence of the mercury resistance genes *merA*/*B* and cadmium resistance (SCC-associated $cadD_{(R35)}$), as well as of the gene encoding surface-anchored protein X, *sasX* (*sesI*; FN433596.1: position 2307955 to 2308569). It lacked enterotoxin genes. The SCC-associated markers (see [15] and Table 4) showed an SCC*mec* pattern as predicted for Bmb9393, GenBank CP005288.1 and CUHK_HK2007, GenBank AZMZ. The remaining five isolates were different, lacking *mer* and *sasX* but harbouring enterotoxin genes *sea*, *sek* and *seq*. Four out of five of these isolates carried multiple *erm* (*ermA* and *ermC*) genes. These isolates yielded a different SCC pattern, matching the prediction for CN79, GenBank ANCJ.

CC772-MRSA

Based on the MLST data, CC772 has been considered as part of CC1 but is regarded here as a CC on its own (see [30]).

Fourteen isolates were assigned to this clonal complex, 13 of which were PVL-positive. One had a deviant SCC*mec* IV + *ccrC* composite element (see Table 4) that also included heavy metal (As, Cd) resistances.

The one PVL-negative isolate also lacked the enterotoxin A gene *sea*, which is normally situated on the same prophage in this strain as PVL. This isolate had the same SCC*mec* V subtype as the "Bengal Bay Clone", but a different one than the related but PVL-negative "WA MRSA-10" (ST573-MRSA-V). Thus, it should be regarded as a phage-deficient variant of the "Bengal Bay Clone", rather than a "WA MRSA-10".

Two isolates that lacked gentamicin/tobramycin resistance (*aacA-aphD*) and that harboured tetracycline resistance (*tetK*) presented with a different SCC*mec* subtype than normally present in the "Bengal Bay" strain. Hybridisation signals were consistent with the SCC*mec* VT element found in the European CC398 livestock-associated MRSA. The remaining ten isolates were assigned to the "Bengal Bay Clone".

Discussion

In this study, we report the genotyping of a series of hospital-acquired MRSA from Pakistan. Molecular typing data from this country are scarce and only a few studies are available that also targeted other patient collectives [24].

A high rate of PVL (34%) needs to be emphasised. This can be nearly exclusively attributed to CC772 (see below). Regarding resistance markers, an absence of *vanA* and *cfr* was noted, but *mupA* was found to be present, albeit still at rather low levels.

The overall population structure of MRSA observed herein showed connections to the Middle East. Strains that are known to be common there include CC5-MRSA-[IV + *fus* + *ccrAB*] "Maltese Clone" [37, 38], CC6-MRSA-IV [34, 38] and CC22-MRSA-IV (*tst1*-positive) "Gaza Clone" [42–45]. A close epidemiological link to the Middle East or Arab Gulf countries in particular can be expected because as much as approximately 3,600,000 Pakistani citizens are currently employed there (see https://tinyurl.com/yax8a33t).

As in a previous study [24], a high number of isolates was assigned to ST772-MRSA-V (PVL+), also known as "Bengal Bay Strain". A single PVL/*sea*-negative isolate was regarded as a deletion mutant of this strain rather than a rare representative of the related ST573-MRSA-V "WA MRSA-10" because its SCC*mec* element was identical to the one from the "Bengal Bay Clone" (see the Results section). The latter strain emerged on the Indian subcontinent, where it is now common. In Western Europe, it is rather frequently encountered in patients with epidemiological links to the Indian subcontinent (travel history, family ties etc.; [26, 27, 30, 32, 33, 46]. It also became common in Middle Eastern/Arab Gulf countries and it needs to be discussed whether it was imported to Pakistan from there rather than directly from India. The notably high prevalence of PVL genes among hospital-acquired MRSA in the described Pakistani setting can be largely attributed to the presence of this strain. In general, this strain appears to be an increasingly important public health threat in several countries in the region, as it can be encountered in hospital as well as in community settings, as it is virulent and carries more diverse resistant determinants than most other community-associated *S. aureus*/MRSA strains. One CC772 presented with a yet unknown SCC*mec* IV composite element and two isolates had SCC*mec* VT + *czrC* elements as observed in the European livestock-associated CC398 strain. We do not have an explanation for this but we noted the same elements in two

Table 4 SCC*mec* elements observed in this study, subtypes, reference sequences and their gene content

SCC*mec* subtype	Matching sequences (accession numbers)	*mec* complex	Heavy metal resistance genes	Other markers	Recombinase genes	SCC termini	Identified in CC
SCC [*mec* II (kdp-) + As/Cd]$_{(Unknown)}$	No matching sequence identified, presumably new SCC*mec* element	*mecA*, *ugpQ*, *mecR*, *mecI*, *psmMEC*, *xylR*	*arsB*, *cadD* $_{(R35)}$	*mvaS*, *cstB*-SCC1, *cstB*-SCC2, *Q93IB7*, *B2Y834*	*ccrA* + *B-2*	*dcs*	CC8
SCC [*mec* III + Cd + *ccrC*] $_{(CN79)}$	ANCJ, BABZ	*mecA*, *ugpQ*, *mecR*, *mecI*, *psmMEC*, *xylR*	*cadD* $_{(R35)}$	*mvaS*, *cstB*-SCC1, D1GU38, Q933A2, D1GU55	*ccrA* + *B-3*, *ccrC*	*dcs*, SCCterm2	CC239
SCC [*mec* III + Cd/Hg + *ccrC*] $_{(Bmb9393)}$	CP005288.1, AZMZ	*mecA*, *ugpQ*, *mecR*, *mecI*, *psmMEC*, *xylR*	*merA/B*, *cadD* $_{(R35)}$	*mvaS*, *cstB*-SCC1, D1GU38, Q933A2	*ccrA* + *B-3*, *ccrC*	SCCterm2	CC239
SCC*mec* IVa $_{(MW2)}$	BA000033.2	*mecA*, *ugpQ*, Delta *mecR1*	–	*mvaS*, *cstB*-SCC2	*ccrA* + *B-2*	*dcs*	CC5, CC8, CC22
SCC*mec* IVa $_{(CMFT503)}$	HF569113.1	*mecA*, *ugpQ*, Delta *mecR1*	–	*mvaS*, *cstB*-SCC2, Q93IB7	*ccrA* + *B-2*	SCCterm1	CC22
SCC [*mec* IVa + ACME1 + Cu] $_{(USA300)}$	CP000255.1, CP000730.1	*mecA*, *ugpQ*, Delta *mecR1*	*copA2*-SCC	*mvaS*, *cstB*-SCC2, *ydhK*, ACME I, *adhC*, *speG*	*ccrA* + *B-2*	*dcs*	CC8
SCC [*mec* IVa + *fus* + *tir* + *ccrAB*] $_{(CMFT181)}$	HF569097.1	*mecA*, *ugpQ*, Delta *mecR1*	–	*mvaS*, *cstB*-SCC2, *fusc*, *tirS*, *yeeA*	*ccrA* + *B-2*, *ccrA* + *B* (see [37])	*dcs*, SCCterm14	CC5
SCC*mec* IVc $_{(IS-105)}$	AHLR	*mecA*, *ugpQ*, Delta *mecR1*	–	B2Y834	*ccrA* + *B-2*	*dcs*	CC8
SCC [*mec* IV + As/Cu + *ccrC*] $_{(Unknown)}$	No matching sequence identified, presumably new SCC*mec* element	*mecA*, *ugpQ*, Delta *mecR1*	*copA2*-SCC, *arsB*, *arsC*	*mvaS*, *cstB*-SCC2, *ydhK*, Q4LAG7	*ccrB-2*, *ccrAA*, *ccrC*	SCCterm11	CC772
SCC [*mec* VT + *czrC*] $_{(SO385)}$	AM990992.1	*mecA*, *ugpQ*	*czrC*	*mvaS*, *cstB*-SCC2, *ydhK*, D1GU38, Q4LAG7	*ccrAA*, *ccrC*	SCCterm2	CC772
SCC*mec* V $_{(Bengal\ Bay)}$	AZBT, ALWH, AJKD, AOCQ	*mecA*, *ugpQ*,	–	*mvaS*, Q4LAG7	*ccrAA*, *ccrC*	SCCterm11	CC772
SCC*mec* VT $_{(H121820035)}$	ERR717021	*mecA*, *ugpQ*,	–	*mvaS*, D1GU38	*ccrC*	SCCterm2	CC22

published CC772 genome sequences (from England and Australia, accession numbers ERR716988 and ERR264123).

A further common strain was ST239-MRSA-III, with two different variants of composite SCC*mec* III/heavy metal resistance elements. This is an ancient, truly multi-resistant and rather strictly hospital-associated strain that has been found in hospitals around the world for decades [11, 47]. It has been previously found in Pakistan, so it is no surprise that it was detected during the present study. The absence from a previous Pakistani study [24] might be attributed to patient inclusion criteria, as that study targeted mainly community-associated cases. One *merA*/*B*- and *sasX*-positive ST239-MRSA-III isolate showed an SCC*mec* pattern as predicted for Bmb9393, GenBank CP005288.1 and CUHK_HK2007, GenBank AZMZ. This might place this isolate into a clade within ST239 together with South American, Portuguese, South-East Asian and Southern Chinese isolates [47]. The more common *merA*/*B*- and *sasX*-negatives yielded SCC signals as predicted for, among others, CN79, GenBank ANCJ or 16K, GenBank BABZ. This places them into a lineage known to occur in Turkey, Eastern Europe, Russia and Northern China [47–49]. These observations might indicate multiple, and independent, importations of ST239-III strains into Pakistan.

Regarding other pandemic clones, one isolate of the PVL/ACME-positive CC8-MRSA-IV strain "USA300" was identified. "USA300" is predominant in the United States but also spread to other, especially Anglophone, countries. Here, again, an importation with returning expatriates and/or an epidemiological link to the USA, UK or some of the Gulf States [11, 50] might be the most likely explanation for this strain's presence in Pakistan. Besides this isolate, there were other CC8-MRSA-IV with varying enterotoxin gene carriage and diverse SCC*mec* IV subtypes. One isolate was PVL-positive but differed from "USA300" in the absence of ACME and of an SCC-associated copper resistance gene. It presented with a plain SCC*mec* IVa element, as in MW2 and as in the similar CC8-MRSA-IV strain IS-88, GenBank AHLO. Thus it is also not identical to the "Spanish/Latin American" PVL-positive/ACME-negative CC8-MRSA-IV strain, which harbours an SCC*mec* IVc composite element (strain MRSA177, GenBank AECP). Given the paucity of data from Central Asia, it cannot currently be determined whether this strain, as well as the four PVL-negative CC8-MRSA strains, emerged locally or might have been imported from neighbouring countries. However, it should be noted that CC8-MRSA-IV was also common in an earlier study from Pakistan [23].

One isolate was identified as (*tst1*-negative) CC22-MRSA-IV, thus closely resembling the internationally known "UK-MRSA-15" clone (in Germany, "Barnim Epidemic Strain", named for the county, close to Berlin, where it was first observed [51]). However, SCC*mec* subtyping revealed the presence of an MW2-like SCC*mec* IVa element, while "UK-MRSA-15" have SCC*mec* IV h/j elements such as in HO 5096 0412, GenBank HE681097.1. Thus, it was not a European UK-15/"Barnim" isolate. Since the "Gaza Clone" (see above and Tables 3 and 4) harbours a different, CMFT503-like SCC*mec* IVa subtype, this isolate can also not be considered a *tst1* deletion variant of that strain.

Conclusion

Hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) is a major issue in Pakistan. Dominating strains include variants of the ancient pandemic strain ST239-MRSA-III (that was most likely imported at least twice, as there were two distinct variants), the Panton–Valentine leucocidin (PVL)-positive CC772-MRSA lineage (that was originally community-associated), as well as different CC8-MRSA-IV. The pandemic "USA300" strain can also be found, albeit rarely.

Many of the strains detected herein indicate epidemiological links to the Middle Eastern/Arabian Gulf region.

Further studies are needed to type MRSA from countries with less known epidemiology, such as Pakistan, and to monitor the distribution and spread of multi-resistant bacteria, as well as possible links to global travel, migration and commerce.

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Compliance with ethical standards

Conflict of interest Darius Gawlik, Elke Müller, Annett Reissig, Ralf Ehricht and Stefan Monecke are employees of Abbott (Alere Technologies GmbH), Jena, the company that manufactures the microarrays used for this study.

Informed consent and ethics approval Ethic committees consent was not requested since isolates were not purposefully obtained for this study but retrospectively selected after performing routine diagnostics.

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