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ORIGINAL ARTICLE

Molecular characterization of methicillin-resistant *Staphylococcus aureus* in nosocomial infections in a tertiary-care facility: emergence of new clonal complexes in Saudi Arabia

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Abstract

Changes in the molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) continue to be reported. This study was carried out to characterize MRSA isolates in Saudi Arabia. MRSA isolates causing nosocomial infections (n = 117) obtained from 2009–2015 at a tertiary-care facility in Riyadh, Saudi Arabia, were studied. Molecular characterization of isolates was carried out using the StaphyType DNA microarray (Alere Technologies, Jena, Germany). Fourteen clonal complexes (CC) were identified, with the most common being CC80 (n = 35), CC6 (n = 15), CC5 (n = 13) and CC22 (n = 12). With the exception of nine ST239 MRSA-III isolates, all others were of community-associated MRSA lineages. The following strains are identified for the first time in Saudi Arabia: ST8-MRSA-IV [PVL$^+$/ACME$^+$], USA300 (n = 1); ST72-MRSA-IV USA700 (n = 1); CC5-MRSA-IV, [PVL$^+$/edinA$^+$], WA MRSA-121 (n = 1); CC5-MRSA-V+SCCfus, WA MRSA-14/109 (n = 2), CC97-MRSA-IV, WA MRSA-54/63; CC2250/2277-MRSA-IV and WA MRSA-114. CC15-MRSA (n = 3) was identified for the first time in clinical infection in Saudi Arabia. None of the isolates harboured vancomycin resistance genes, while genes for resistance to mupirocin and quaternary ammonium compounds were found in one and nine isolates respectively. Fifty-seven isolates (48.7%) were positive for Panton-Valentine leukocidin genes. While the staphylokinase (*sak*) and staphylococcal complement inhibitor (*scn*) genes were present in over 95% of the isolates, only 37.6% had the chemotaxis-inhibiting protein (*chp*) gene. Increasing occurrence of community-acquired MRSA lineages plus emergence of pandemic and rare MRSA strains is occurring in our setting. Strict infection control practices are important to limit the dissemination of these MRSA strains.
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Introduction

A shift in the epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) has been demonstrated, with reports of community-acquired (CA) MRSA strains causing nosocomial infections [1]. Published literature from the Arabian Gulf region has shown the spread of CA-MRSA into the healthcare setting, with differences in the predominant strains reported from countries in the region [2–5]. However, there are few data on the molecular characterization of MRSA isolates in Saudi Arabia, where a different population structure of MRSA might be expected as a result of the large number of pilgrims who visit the country annually; the large immigrant workforces from the Indian subcontinent, Maghreb countries, Egypt and Yemen; and a relative absence of Northern American and European tourists [6–8].

We carried out molecular characterization of MRSA isolates causing nosocomial infections in a hospital in Saudi Arabia. The findings indicate emergence of new MRSA clonal complexes.

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Methods

Specimen collection and bacterial strains

The study was carried out at the King Khalid University Hospital in Riyadh, Saudi Arabia. Ethical approval was obtained from the hospital ethics committee. Archived MRSA isolates associated with clinical infection identified from 2009 to 2015 were studied. Bacterial identification and antibiotic susceptibility testing were performed using standard laboratory techniques and the automated MicroScan Walkaway 96 plus System (Siemens Healthcare Diagnostic) in accordance with Clinical and Laboratory Standards Institute guidelines [9]. Methicillin resistance was confirmed on Mueller-Hinton agar using the disk diffusion method to demonstrate resistance to oxacillin and 30 mg cefoxitin disks [9].

Array procedures

Molecular characterization was carried out using the StaphyType DNA microarray (Alere Technologies, Jena, Germany). The included target genes, primer and probe sequences and procedures have been published previously [10]. Briefly, MRSA isolates were collected from Columbia blood agar, and DNA extraction was carried out by enzymatic lysis [10] followed by purification using a commercially available kit utilizing spin columns (Qiagen, Hilden, Germany). The purified DNA was used as template in a linear primer elongation reaction, during which biotin-16-dUTP was incorporated into the resulting amplicons and the single-stranded DNA products were hybridized stringently to the microarray. Incubation with horseradish peroxidase–streptavidin conjugate, which binds to biotin labels, was carried out, and a dye was added which precipitated in the presence of the peroxidase, resulting in the formation of visible spots where hybridization had occurred. Microarray images were taken and analysed with a dedicated reader and software (Alere Technologies). Assignment to clonal complexes and sequence types, as well as identification of epidemic strains was carried out fully automatically with the same software module [8,10].

Results

A total of 117 isolates obtained from wound swabs (*n* = 83), blood cultures (*n* = 26), endotracheal aspirates (*n* = 4), sputum (*n* = 2) and urine (*n* = 2) were studied. A majority of the patients were male (*n* = 62), and their mean ± standard deviation age was 34.9 ± 26.1 years. Most of the patients (83%) were Saudi nationals. There was 100% concordance in the phenotypic and genotypic MRSA identification. All isolates were susceptible to vancomycin.

There was a wide clonal diversity, with 14 clonal complexes (CC) being identified. The most common CCs were CC80, CC6, CC5 and CC22 (Table 1). The most prevalent strain (*n* = 35; 29.9%) was CC80-MRSA-IV [PVL^{+}] (European CA-MRSA clone). Although one isolate with a truncated/atypical SCC*mec* element was identified, we did not find Panton-Valentine leukocidin (PVL)-negative variants of CC80. All the 15 CC6 strains carried SCC*mec* IV and were identical to Western Australia (WA) MRSA-51. The 13 CC5 strains belonged, based on hybridization patterns, to four distinct strains, with the predominant one being CC5-MRSA-IV+SCC-*fus* ("Maltese clone") (Table 1). Twelve isolates were identified as CC22, with the Middle Eastern variant CC22-MRSA-IV [*tst1*$^{+}$] being the most prevalent (*n* = 7). The only classical hospital-acquired (HA)-MRSA isolates identified were nine CC8 isolates, which were ST239-MRSA-III+SCCmer (Vienna/Hungarian/Brazilian Clone).

Our data and actual literature indicate the first report of the following strains in Saudi Arabia: ST8-MRSA-IV [PVL^{+}/ACME^{+}], USA300 (*n* = 1); ST72-MRSA-IV USA700 (*n* = 1); CC5-MRSA-IV, [PVL^{+}/edinA^{+}], WA MRSA-121 (*n* = 1); CC5-MRSA-V+SCCfus, WA MRSA-14/109 (*n* = 2); CC97-MRSA-IV, WA MRSA-54/63 (*n* = 1); and CC2250/2277-MRSA-IV, WA MRSA-114. The CC15-MRSA strain (*n* = 3) was identified for the first time in clinical infection in Saudi Arabia. Additionally, a strain was observed that was either a CC22-MRSA-IV [fnbB-,sec/l^{-}], United Kingdom epidemic MRSA-15 (UK-EMRSA-15)/Barnim EMRSA or a *tst1*-negative deletion variant of a strain commonly

TABLE 1. Distribution of MRSA clonal complexes

Clonal complex	Strain affiliation	Total
CC1	CC1-MRSA-IV, WA MRSA-1/57	1
CC5	CC5-MRSA-IV+SCCfus, Maltese clone (*n* = 7)	13
	CC5-MRSA-V+SCCfus, WA MRSA-14/109 (*n* = 3)	
	CC5-MRSA-IV, [PVL^{+}] (*n* = 2)	
	CC5-MRSA-IV, [PVL^{+}/edinA^{+}], WA MRSA-121 (*n* = 1)	
CC6	CC6-MRSA-IV, WA MRSA-51	15
CC8	ST239-MRSA-III+ccrC, Vienna/Hungarian/Brazilian clone (*n* = 9)	11
	ST8-MRSA-IV [PVL^{+}/ACME^{+}], USA300 (*n* = 1)	
	ST72-MRSA-IV, USA700 (*n* = 1)	
CC15	CC15-MRSA (*n* = 1)	3
	CC15-MRSA-V+SCCfus (*n* = 2)	
CC22	CC22-MRSA-IV [fnbB^{+}], UK-EMRSA-15/Barnim EMRSA variant (*n* = 1)	12
	CC22-MRSA-IV [PVL^{+}] (*n* = 4)	
	CC22-MRSA-IV [tst1^{+}],/Middle Eastern variant (*n* = 7)	
CC30	CC30-MRSA-IV [PVL^{+}], Southwest Pacific clone (*n* = 6)	8
	CC30-MRSA-? [VI+fus composite] (*n* = 2)	
CC80	CC80-MRSA-IV [PVL^{+}], European CA-MRSA clone	35
CC88	CC88-MRSA-IV [PVL^{+}]	6
CC97	CC97-MRSA-V [fusC^{+}] (*n* = 7)	8
	CC97-MRSA-IV, WA MRSA-54/63 (*n* = 1)	
CC2250	CC2250/2277-MRSA-IV, WA MRSA-114	2
CC96	CC96-MRSA-IV	1
CC45	CC45/agrIV-MRSA-IV, WA MRSA-23	1
CC913	ST913-MRSA-IV	1
Total		117

MRSA, methicillin-resistant *Staphylococcus aureus*; UK-EMRSA, United Kingdom epidemic MRSA; WA MRSA, Western Australia MRSA.

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TABLE 2. Distribution of virulence and resistance gene markers in 117 isolates

Virulence and resistance genes		Isolates positive for gene n	%
Accessory gene regulator allele I	*agrI*	46	39.3
Accessory gene regulator allele II	*agrII*	17	14.5
Accessory gene regulator allele III	*agrIII*	51	43.6
Accessory gene regulator allele IV	*agrIV*	6	5.1
Alternate penicillin-binding protein 2, defining MRSA	*mecA*	117	100.0
Mercury resistance operon	*merA*	7	6.0
	merB	7	6.0
SCC*mec* XI	*mecC*	0	0.0
	blaZ-SCCmec XI	0	0.0
β-Lactamase	*blaZ*	105	89.7
β-Lactamase repressor (inhibitor)	*blaI*	105	89.7
β-Lactamase regulatory protein	*blaR*	105	89.7
Rrna adenine N-6-methyl-transferase, erythromycin/clindamycin resistance	*ermA*	10	8.5
Erythromycin/clindamycin resistance	*ermB*	0	0.0
Erythromycin/clindamycin resistance	*ermC*	21	17.9
Linco-samid-nucleo-tidyltrans-ferase	*linA*	6	5.1
Energy-dependent efflux of erythromycin	*msrA*	5	4.3
Bifunctional enzyme Aac/Aph, gentamicin resistance	*aacA-aphD*	14	12.0
Amino-glycoside adenyl-transferase, tobramycin resistance	*aadD*	10	8.5
3′5′-Aminog-lycoside phospho-transferase, neo-/kanamycin resistance	*aphA3*	42	35.9
Strepto-thricine-acetyl-transferase	*sat*	42	35.9
Dihydro-folate reductase type I	*dfrA*	9	7.7
Fusidic acid resistance	*far1*	34	29.1
Hypothetical protein associated with fusidic acid resistance	*Q6GD50 (fusC)*	23	19.7
Mupirocin resistence protein	*mupR*	1	0.9
Tetracycline-resistance	*tetK*	31	26.5
Tetracycline-resistance	*tetM*	10	8.5
Chloramphenicol acetyltransferase	*cat*	2	1.7
23S rRNA methyltransferase	*cfr*	0	0.0
Chloramphenicol/florfenicol exporter	*fexA*	1	0.9
Metallothiol transferase	*fosB*	52	44.4
	fosB-plasmid	7	6.0
Quaternary ammonium compound resistance protein A	*qacA*	9	7.7
Quaternary ammonium compound resistance protein C	*qacC*	0	0.0
Transport/efflux protein	*tetEfflux*	103	88.0
Vancomycin resistance gene	*vanA*	0	0.0
Vancomycin resistance gene from enterococci and *Clostridium*	*vanB*	0	0.0
Teicoplanin resistance gene from enterococci	*vanZ*	0	0.0
Toxic shock syndrome toxin I	*tst1 (consensus)*	10	8.5
Panton-Valentine leukocidin F component	*lukF-PV*	57	48.7
Panton-Valentine leukocidin S component	*lukS-PV*	57	48.7
Staphylokinase	*sak*	113	96.6
Chemotaxis-inhibiting protein	*chp*	44	37.6
Staphylocccal complement inhibitor	*scn*	116	99.1
Exfoliative toxin serotype A	*etA*	1	0.9
Exfoliative toxin serotype B	*etB*	0	0.0
Exfoliative toxin D	*etD*	36	30.8
Epidermal cell differen-tiation inhibitor	*edinA*	2	1.7
Epidermal cell differentiation inhibitor B	*edinB*	36	30.8
Epidermal cell differentiation inhibitor C	*edinC*	0	0.0
Arginine catabolic mobile element locus	*ACME*	1	0.9

MRSA, methicillin-resistant *Staphylococcus aureus*.

observed in the Middle East ("Gaza epidemic strain") (*n* = 1) (Table 1), thus warranting further investigations on differentiation and molecular epidemiology of CC22 MRSA.

Table 2 shows the distribution of resistance and virulence genes. The most predominant accessory gene regulator alleles were *agrI* and *agrIII*. None of the isolates harboured the SCC*mec* XI and/or the *mecC* gene. A majority of isolates (*n* = 105; 89.7%) carried the β-lactamase operon. Twenty-three isolates (19.7%) carried the fusidic acid resistance gene *fusC* (Q6GD50). Only one isolate had the mupirocin resistance gene (*mupR*), and the gene encoding the quaternary ammonium compound resistance protein A (*qacA*) was found in nine isolates (7.7%). Almost half of the isolates were PVL positive (*n* = 57; 48.7%). While the staphylokinase (*sak*) and staphylococcal complement inhibitor (*scn*) genes were present in over 95% of the isolates, only 37.6% had the chemotaxis-inhibiting protein (*chp*) gene (Table 2). The arginine catabolic mobile element (ACME) locus was present in only one isolate (CC8-MRSA-IV, "USA300").

Discussion

An increasing trend of MRSA endemicity has been reported in healthcare facilities in Saudi Arabia, with CA-MRSA contributing to the burden of infections [8]. However, there are few data describing the MRSA clonal complexes in Saudi Arabia [6–8]. A 2005 report showed the predominance of the hospital-acquired lineage ST239 MRSA-III [7]. Using DNA microarray-based typing, a high diversity of clonal complexes with predominance of CC8/ST239-III, CC22-IV, CC30-IV and CC80-IV was demonstrated in 2011–2012 in a tertiary-care hospital in Riyadh [8]. The findings from our study also show a high diversity in MRSA and suggest a potential for rapid changes. This is rather different than the situation in the United States, where "USA300" dominates, or the Western Europe, where PVL-negative CC22-MRSA-IV (UK-EMRSA-15/Barnim EMRSA) are most abundant [1]. In addition, our findings confirm that a shift has occurred, with what are presumably CA-MRSA clonal lineages now predominating within the healthcare, setting as majority of the isolates harboured SCC*mec* IV and V [6,8]. Of significance, however, is that we now report the first identification of new MRSA strains, including rare and pandemic ones, in Saudi Arabia.

CC5

In this study, CC5-MRSA-IV+SCC*fus,* Maltese clone strain, was the predominant CC5. This strain, which was originally identified in Malta, harbours the fusidic acid resistance marker Q6GD50 (*fusC*), SCC*mec* IV and additional recombinase genes [11]. As it has only been reported in one study in Saudi Arabia, our finding represents the second reported identification in the country and the region [8]. Similar to previous report from Saudi Arabia, we also identified CC5-MRSA-IV, [PVL^{+}] (paediatric clone, or USA800) [8]. This has been identified as the second most predominant MRSA strain in Qatar [5]. Three variants of PVL-positive CC5-MRSA-IV based on combination of virulence genes have been described [10]. The WA MRSA-121 is one of these variants, as it is a PVL-positive CC5-

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MRSA-IV strain harbouring the *edinA* gene and has not been previously identified in our region. CC5-MRSA-V has been described in Australia, Ireland and the United Arab Emirates [10]. Our identification of two isolates designated as CC5-MRSA-V [*fusC*$^+$] represents the first description of an isolate very similar to WA MRSA-14 in Saudi Arabia.

CC6

All 15 CC6 strains were identical to WA MRSA-51 [12]. This is similar to previous work in Riyadh in which all the CC6 strains were WA MRSA-51 strains and represents its second description in Saudi Arabia [8]. This strain has been described in Australia and United Arab Emirates [10,12]. CC6 methicillin-susceptible *S. aureus* (MSSA) has been reported as a common clone in Middle Eastern camels; however, only one isolate was identified in nasal colonization isolates in Riyadh [10,13].

CC8

A majority of the CC8 isolates belonged to the ST239-MRSA-III+SCC*mer* (Vienna/Hungarian/Brazilian clone). Our findings support recent data which indicates that this HA-MRSA strain, which has been described as the oldest pandemic MRSA strain, has been displaced by CA-MRSA strains in the hospital environment in Saudi Arabia [8]. However, in a departure from the 2012 report by Monecke *et al.* [8] in which all CC8 isolates were ST239, our findings indicate the appearance of two other CC8 strains. We present the first report of the pandemic USA300 (ST8-MRSA-IV [PVL$^+$/ACME$^+$]) and USA700 (ST72-MRSA-IV) in Saudi Arabia. USA300 is the dominant MRSA strain in the United States, and although it has been identified in Europe, the occurrence is much less frequent there [14,15]. In our region, this strain has been described in the United Arab Emirates and Qatar [5,10]. The ST72 strain belongs to CC8 according to the MLST (multilocus sequence typing) database, but it typically yields a different hybridization profile compared to other CC8 strains. It has been suggested that this strain probably arose as consequence of multiple recombination events of the CC5 and CC8 genomes [10]. Our isolate was USA700, which is a PVL-positive ST72-MRSA-IV strain. In our region, it had been identified in the United Arab Emirates, and our finding represents its first identification in Saudi Arabia [10].

CC15

Although CC-15-MSSA is ubiquitous among healthy carriers, CC15 MRSA remains rare [10]. Recent work from Riyadh, Saudi Arabia, found CC15-MSSA as the predominant nasal colonization MSSA isolate [13]. CC-15 MRSA isolates have been identified in few reports [6,16,17]. Two CC-15 MRSA isolates which were nasal colonizers were reported from Iran and Saudi Arabia [6,17]. Our findings therefore indicate the first report of CC-15 MRSA isolate causing nosocomial infection in the region.

CC22

Similar to a previous report from Saudi Arabia, both PVL-positive and PVL-negative CC22 strains were identified [8]. The CC22-MRSA-IV UK-EMRSA 15 is a pandemic PVL-negative strain which has very high incidence of 50–95% in Western Europe and has also been described in Malta, Kuwait, the United Arab Emirates and Qatar [5,10,18]. There is also a Middle Eastern variant that differs from the true UK-EMRSA-15 in its SCC*mec* subtype (SCC*mec* IVa rather than IVh/j) and in the presence of the *tst1* gene. Our finding echoes previous reports of the identification of this strain in other reports from the region [10]. PVL-positive CC22 MRSA-IV has been described in Germany, Australia, England, Ireland, Hong Kong and the United Arab Emirates [10]. This strain might be widespread in the region, as they were also described in a previous study from Saudi Arabia and in German patients with ties to Turkey [8,10].

CC30

Six isolates belonged to the CC30-MRSA-IV [PVL$^+$], Southwest Pacific clone (USA1100). This strain is widely distributed in the Pacific islands and was first identified in New Zealand among the Samoan immigrant population [10]. It is a widespread MRSA clone and has been found in several European countries as well as in Hong Kong, Taiwan and the United States [10]. In our region, it has been described in Saudi Arabia, Kuwait, the United Arab Emirates, Oman and Qatar [3,5,8]. The occurrence of this strain in our region could possibly be linked to the large population of expatriates from the Pacific region, in particular the Philippines. In this study we identified a previously undescribed CC30-MRSA-VI/SCC*fus* strain which was PVL-positive. This strain differs from other known CC30 strains in its carriage of SCC*mec* type VI with the fusidic acid resistance gene marker.

CC80

The majority of our isolates were PVL-positive CC80-MRSA IV (European CA-MRSA clone), which is in accordance with previous reports from Saudi Arabia and from other countries in the region [5,10,19,20]. This strain has also been shown to be predominant in North Africa, which suggests a spread from Europe into North Africa and the Middle East [21–23]. Conversely, this strain could have spread from these regions into Europe as a consequence of travel to Turkey and the Maghreb countries (which are both popular holiday destinations and the homelands of large immigrant communities in

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Europe) [10]. In contrast to reports from France and Croatia as well as a previous report from Saudi Arabia, no PVL-negative variant of CC80 was identified in our study [8,24]. However, we identified one variant, CC80-MRSA, which was PVL positive, which had a truncated/atypical SCC*mec* element lacking *ccr* genes.

CC88

CC88-MRSA-IV strains carrying the exfoliative toxin gene *etA* have been described in the Netherlands, Portugal, Angola, Senegal and Japan [10]. The CC88-MRSA-IV strains we identified in this study were PVL positive but lacked the *etA* gene. This is in contrast to a previous report from Saudi Arabia in which the CC88-MRSA-IV [PVL$^+$] identified harboured the *etA* gene [8].

CC97

This MRSA clonal complex has only been sporadically identified in reports from Australia, France, the United Arab Emirates, Kuwait and Egypt [10,25]. Seven of the eight CC97 strains in this study were CC97-MRSA-V [*fusC*$^+$] and were similar to those previously identified in Saudi Arabia [8]. One CC97-MRSA belonged to CC97-MRSA-IV (corresponding to WA MRSA-54/63), which had not previously been described in Saudi Arabia.

Two isolates of CC2250/2277-MRSA-IV, WA MRSA-114, and one each of CC1-MRSA-IV, WA MRSA-1/57, CC45/agrIV-MRSA-IV, WA MRSA-23 and CC913 were identified. The CC1 isolate had SCC*mec* IV, lacked the SCC*fus* and was PVL negative. CC1-MRSA isolates with these characteristics included WA MRSA-57 and WA MRSA-1 [10]. The strain affiliation for our isolate is therefore regarded as CC1-MRSA-IV, WA MRSA-1/57, which has been described in Germany, Ireland and Egypt [10]. This strain appears infrequent in our setting; only one was identified in previous work in Saudi Arabia [8]. The CC45/*agr*IV-MRSA-IV, WA MRSA-23 strain, which has been linked to Hong Kong, South China and Australia, has previously been reported in Saudi Arabia [8,10]. Our report of CC2250/2277-MRSA-IV, WA MRSA-114 marks the first description of the *Staphylococcus argenteus* lineage MRSA in Saudi Arabia. CC913 represents a rare group which had previously been identified in a Lebanese immigrant in Germany and in subjects from the Negev region [10]. This first identification in our setting supports the notion of a wider distribution of this lineage in the Middle East.

The *PVL* genes (*lukF-PV+lukS-PV*) have been implicated as playing a role in the development of skin and soft tissue infections. Similar to our findings, high numbers of PVL-positive strains have been reported from Algeria and the United Arab Emirates [10,26]. However, our findings are higher compared to reports from some European countries [10,15,27,28]. Previously published data from Saudi Arabia showed a lower PVL prevalence, ranging from 8% to 19% [29]. Reasons for this difference are largely unknown; it could be speculated that these PVL-positive MRSA have emerged from PVL-positive MSSA. However, if this were the case, then frequency of PVL-positive MSSA should also be high in our population. Recent data on MSSA isolates from nasal colonization in Riyadh showed very low prevalence of *PVL* genes [13]. A number of other virulence genes were detected in different proportions. A high prevalence of the staphylococcal complement inhibitor (*scn*) and staphylokinase (*sak*) were observed. However, chemotaxis-inhibiting protein (*chp*) was identified in fewer isolates. These virulence genes have also been identified as being widely distributed in MSSA associated with nasal colonization in Saudi Arabia [13].

Although studies have shown an increasing trend of phenotypic resistance to various antibiotics in CA-MRSA isolates from Saudi Arabia, no vancomycin resistance has yet been reported [30]. However, there are few data on the distribution of resistance genes [8]. The DNA microarray used in this study enabled us to describe a wide spectrum of resistance and virulence genes. Our findings are in accordance to previously reported work using this methodology to characterize MRSA isolates from Saudi Arabia [8]. To better map the evolution of resistance genes in MRSA isolates in our setting, further work using robust methodologies such as the DNA microarray approach are needed.

A limitation of this work is that it was carried out in a single healthcare facility. However, the findings highlight the urgent need for multicentre studies to elucidate the population structure of MRSA in Saudi Arabia. Strict adherence to infection control practices and continued surveillance using improved technology are advocated.

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Conflict of Interest

RE and SM are employees of Alere Technologies GmbH, but this did not influence the study design, and they have no competing interests to declare. None of the other authors has any financial or other relationships that may constitute a conflict of interest.

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