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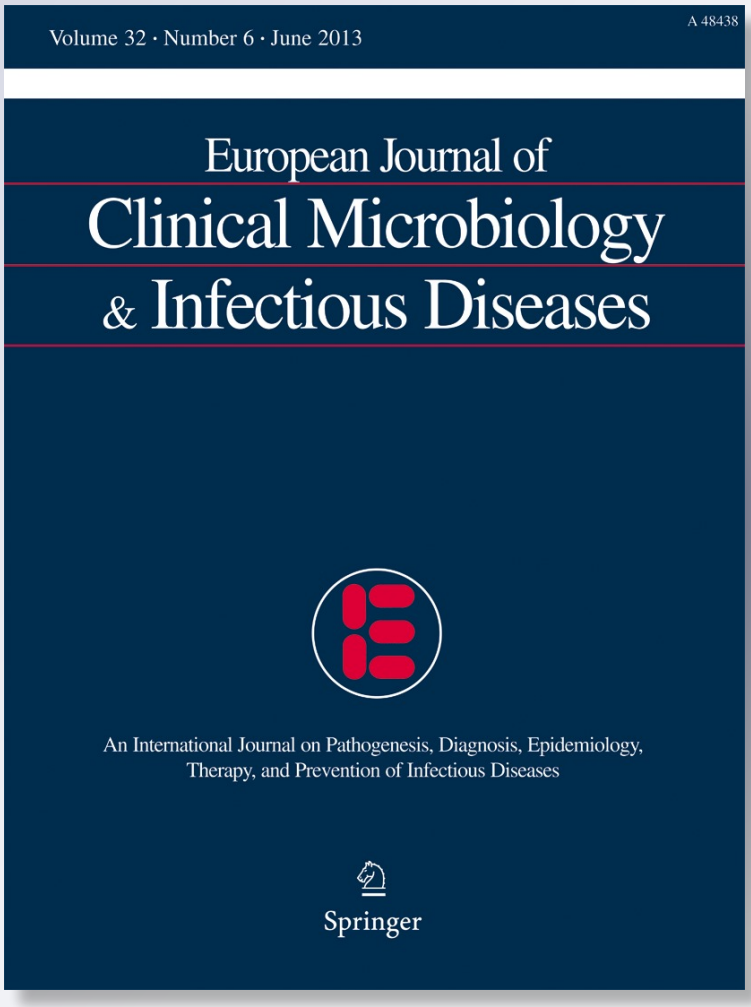

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ARTICLE

Genetic variation among methicillin-resistant *Staphylococcus aureus* isolates from cancer patients in Saudi Arabia

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Abstract One hundred and twenty methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from cancer and non-cancer patients in Saudi Arabia were investigated for antibiotic resistance, virulence determinants and genotypes. The majority of MRSA isolates from cancer (n=44, 73.3 %) and non-cancer patients (n=34, 56.7 %) were multi-resistant to more than four classes of antibiotics. Virulence gene profiling showed that all strains were commonly positive for adhesin genes, except *ebps* and *bbp* genes, which were not detected in any isolate. Although the presence of adhesin genes varied slightly among MRSA isolates from cancer and non-cancer patients, these variations were not found to be statistically significant. In contrast, the presence of the toxin genes *seb*, *sec*, *seg* and *sei* was significantly elevated in MRSA strains isolated from cancer patients. Multilocus sequence typing (MLST) detected six and nine sequence types (STs) among isolates from cancer and non-cancer patients, respectively. Using *spa* typing, 12 and 25 types were detected, including four new types. The ability of different MRSA clones to become multi-resistant and their ability to acquire different virulence factors may contribute to their success as pathogens in individual groups of patients.

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Introduction

Since its discovery during the 1880s, *Staphylococcus aureus* has emerged as a potentially pathogenic Gram-positive bacterial species that may cause various infections such as bacteraemia, infective endocarditis, sepsis, toxic shock syndrome, and skin and soft tissue infections. Following the introduction of antibiotics, methicillin-resistant *S. aureus* (MRSA) have emerged after the acquisition of a mobile genetic element called the staphylococcal chromosomal cassette (SCC) *mec*, which carries the β-lactam resistance gene *mecA*.

The ability of MRSA to cause a spectrum of human diseases is due to a combination of largely unknown host factors and bacterial virulence factors, including cell-surface-associated adhesins and secreted toxins. The type as well as severity of MRSA infection and response to antibiotic treatment are dictated by virulence and resistance genes [1]. There are over 40 virulence-associated genes that have been identified among strains of MRSA, rendering the molecular basis of MRSA infection a complex one [2].

There has been a number of reports concerning risk factors for MRSA infection. Risk factors that have been associated with MRSA acquisition include age, prolonged hospitalisation, prior antibiotic therapy, severe underlying disease and degree of disability, surgical procedures, intravascular devices, mechanical ventilation, impairment of local defence and compromised host immunity [3]. Interestingly, cancer patients may have multiple predisposing factors that increase the risk of infection. Chemotherapy, radiation therapy, surgery, stem cell transplantation, bone marrow transplantation or steroids in addition to the cancer itself can suppress or

weaken the immune system [4–6]. Since individual strains of MRSA differ from one another in the arrangement of chromosomal alleles and in the content of variable accessory genetic elements, the elucidation of genetic characteristics of the local MRSA strains is necessary as a basis for efficient infection control.

Although some MRSA genotypes from Saudi Arabia have been reported [7, 8], this study provides the first data on MRSA genotypes in cancer patients from the setting of one of the largest hospitals in Saudi Arabia. Knowledge of the antibiotic resistance, genotypic and virulence genes profiles of pathogenic MRSA may provide advantages in controlling MRSA infections in cancer patients undergoing therapy.

Materials and methods

Isolates

A total of 120 infectious MRSA isolates were obtained from the Medical Microbiology Laboratory, Riyadh Armed Forces Hospital. Among these isolates, 60 isolates were obtained from cancer patients and another 60 isolates from non-cancer patients. Samples were collected between February and August 2010. *S. aureus* was identified from clinical specimens on the basis of colony appearances, coagulase and catalase tests. The resistance to methicillin was determined by the disc diffusion test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [9] and polymerase chain reaction (PCR) detection of the *mecA* gene [10].

Antibiotic susceptibility

Antimicrobial susceptibility tests were performed by the disc diffusion method as recommended by the CLSI [9]. Thirteen antimicrobial agents were tested: oxacillin (1 μg), cefoxitin (30 μg), penicillin (1 μg), ampicillin (10 μg), amoxicillin (1 μg), cotrimoxazole (25 μg), cephalothin (30 μg), erythromycin (15 μg), clindamycin (2 μg), fusidic acid (10 μg), mupirocin (5 μg), rifampicin (30 μg) and vancomycin (30 μg). *S. aureus* ATCC 25923 was used as the control strain. Susceptibility to fusidic acid was interpreted following guidelines by the British Society for Antimicrobial Chemotherapy (BSAC) [11].

DNA isolation

Genomic DNA was extracted using the GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia), according to the manufacturer's instructions. The purity and concentration of DNA were measured using the Eppendorf BioPhotometer Plus analysis system (Eppendorf, Germany). The extracted DNA was used as the template in all PCR assays.

SCC*mec* typing

SCC*mec* types were determined using multiplex PCR, as described previously [12]. As all isolates in this study were characterised as hospital-acquired MRSA (HA-MRSA), only three SCC*mec* types, I–III, were investigated.

spa typing

Amplification of the *spa* repeat region was performed using *spa*-f (5-AAAGACGATCCTTCGGTGAGC-3) and *spa*-r (5-CAGCAGTAGTGCCGTTTGCTT-3) [13]. PCR products were purified using the GF-1 PCR Clean-up Kit (Vivantis Technologies, Malaysia), according to the manufacturer's instructions and sequenced commercially (First BASE Laboratories Sdn Bhd, Malaysia). The sequences were subjected to *spa* repeat analysis. *spa* types (t) were determined by using the Ridom StaphType software (Ridom GmbH, Germany). By applying the recently developed algorithm BURP (Based Upon Repeat Patterns), *spa* types were clustered and the clonal complexes (*spa* CCs) were automatically assigned by this software.

MLST

Multilocus sequence typing (MLST) was performed only when a sequence type (ST) was not obtained from the *spa* typing database, as previously described [14]. The PCR products of seven housekeeping genes were purified and sequenced. The sequence of each gene was entered on the MLST website (http://saureus.mlst.net), where the allelic profiles were defined assigned to a ST. STs were assigned to CCs using the clustering algorithm eBURST (Based Upon Related Sequence Types). Isolates that do not share alleles at six of the seven MLST loci with any ST in the MLST database were deemed singletons.

Virulence gene detection

The following genes were examined using PCR assays: *fnbA*, *fnbB*, *fib*, *clfA*, *clfB*, *eno*, *cna*, *bbp*, *ebpS*, *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *tst*, *etA*, *etB* and *pvl*. The detection of these genes was achieved via previously described methods [2, 15]. The Panton–Valentine leucocidin (*pvl*) gene was detected using the primers previously described [16]. PCR was performed independently for each gene. Positive and negative controls were included in each PCR run. Amplicon sequences were verified against GenBank database sequences to ensure specificity.

Statistical analysis

The data were analysed using the statistical software package SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). A *p*-value less than 0.05 indicated statistical significance.

Results

Antibiotic susceptibility testing

Antimicrobial susceptibility testing revealed ten resistance profiles. Nine and seven antibiotic susceptibility profiles were observed among the MRSA isolates from cancer and non-cancer patients, respectively (Table 1). All isolates (100 %) were resistant to the β-lactams oxacillin, cefoxitin, penicillin, amoxicillin and cephalothin. High rates of resistance were observed against cotrimoxazole (100 %), fusidic acid (76.7 % and 70 %), erythromycin (70 % and 60 %), clindamycin (41.7 % and 36.7), rifampicin (33.3 % and 35 %) and mupirocin (33.3 % and 35 %) from cancer and non-cancer patients, respectively. Multi-resistance was observed in 41 (68.3 %) MRSA isolates from cancer patients and in 30 (50 %) MRSA isolates from non-cancer patients (p=0.041).

SCC*mec* typing

The *pvl* gene was 100 % absent but the SCC*mec* type III (and, hence, *mecA*) was detected in all (100 %) isolates. None of them showed amplification for SCC*mec* types I and II.

Distribution of MRSA *spa* types

A total of 30 different *spa* types was detected. Twelve and 25 were detected in cancer and non-cancer patients, respectively. Six *spa* types, t037, t030, t044, t690, t363 and t304, were detected among both groups (Table 2). Of these *spa* types, the most prevalent *spa* types were *spa* types t037 and t041, with 42 and 23 occurrences in this study, respectively. *spa* types t030 and t363 occurred six times, and types t002 and t304 occurred five times, whereas t631 and t690 occurred three times each.

Among the MRSA isolates from non-cancer patients, four *spa* types were identified as novel (local *spa* types) after synchronisation with the Ridom database and identified as t7604, t8506, t8507 and t8855. One of the new *spa* types, t7604 (13 repeats), together with *spa* types t032 (16 repeats) and t5507 (13 repeats) were among the longest ones identified. *spa* types were clustered into seven groups, which were assigned as *spa* CCs, non-founders or singletons *spa* types (Table 2).

MLST

Six and nine STs were detected among isolates from cancer and non-cancer patients, respectively. Five STs were detected among both groups (Table 3). The application of eBURST showed that the isolates belonged to two different clonal clusters and three singletons. ST239, ST241 and ST08 were clustered in group 1, whereas ST22 and ST217 were clustered in group 2. Five STs, ST182, ST71, ST88, ST30 and ST80, were identified as singletons. Five STs were detected in both groups, whereas four STs were detected only in non-cancer patients (Table 3).

Virulence gene profiles of MRSA strains

All MRSA isolates from cancer and non-cancer patients were positive for *fnbA*, *clfA*, *clfB*, *fib* and *eno*. *fnbB* and *cna* were detected in 36 and 35 MRSA isolates from cancer and in 25 and 33 MRSA isolates from non-cancer patients, respectively. For the toxins, 68.3 % of the MRSA isolates

Table 1 Antibiotic susceptibility profiles of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates

No.	Resistance profile	Non-cancer, *n* (%)	Cancer, *n* (%)
1	OXA FOX AMX AMP CEP PEN TS	16 (26.7)	9 (15)
2	OXA FOX AMX AMP CEP PEN TS FUS	10 (16.6)	6 (10)
3	OXA FOX AMX AMP CEP PEN TS ERY	3[1] (5)	3 (5)
4	OXA FOX AMX AMP CEP PEN TS ERY FUS	10 (16.6)	8 (13.3)
5	OXA FOX AMX AMP CEP PEN TS CLI		2 (3.3)
6	OXA FOX AMX AMP CEP PEN TS CLI ERY FUS	1 (1.7)	11 (18.3)
7	OXA FOX AMX AMP CEP PEN TS CLI ERY		1 (1.7)
8	OXA FOX AMX AMP CEP PEN TS FUS MUP RIF		1 (1.7)
9	OXA FOX AMX AMP CEP PEN TS CLI ERY MUP RIF	1 (1.7)	
10	OXA FOX AMX AMP CEP PEN TS CLI ERY FUS MUP RIF	18 (30)	18 (30)

Table 2 *spa* typing BURP (Based Upon Repeat Patterns) cluster analysis of MRSA strains

Cluster group and *spa* CC	*spa* type	Cancer ($n=60$)	Non-cancer ($n=60$)	Total no. of strains (%)	Total no. of *spa* types (%)
Cluster 1 *spa* CC 037	t019		2	66 (55)	11 (36.6)
	t030	3	3		
	t037	29	13		
	t138		1		
	t363	1	5		
	t388		2		
	t459		1		
	t631		3		
	t748	1			
	t932	1			
	t1070		1		
Cluster 2 *spa* CC 790	t032	2		9 (7.5)	7 (23.3)
	t223	2			
	t790		1		
	t4573		1		
	t7604		1		
	t8506		1		
	t8855		1		
Cluster 3 *spa* CC 376	t044	13	10	25 (20.8)	3 (10)
	t376		1		
	t8731		1		
Cluster 4 *spa* CC 690	t690	2	1	5 (4.1)	3 (10)
	t729		1		
	t8507		1		
Cluster 5 no founder	t304	2	3	6 (5)	2 (6.6)
	t701		1		
Singletons	t002		5	9 (7.5)	4 (13.3)
	t364	1			
	t2235		1		
	t3059	2			

The **bold font** shows new *spa* types detected in this study

harboured at least one enterotoxin gene. The most prevalent enterotoxin genes were *sei* (35) and *seb* (19), followed by the *seg* (16), *sea* and *tst* (9), and *sec* (6) genes. The same genes except for *tst* were detected but with lower prevalence rates, *sei* (30), *sea* (3), *seb* and *sec* (1), and *seg* (3), among the isolates from non-cancer patients. None of the isolates harboured *sed*, *see*, *eta* or *etb* genes.

Twenty-six of the MRSA harboured more than one superantigen gene, and different gene combinations were observed. Eighteen isolates were positive for both *seb* and *sei* genes. Five isolates were positive for both *sea* and *seb*, and two isolates were positive for *sea*, *seb* and *sec* genes. The combination of *sea* and *sec* was detected in three isolates, whereas *sec* and *seb* were detected together in five isolates. The *seg* virulence determinant was always associated with the *sei* gene present in 19 of the tested isolates.

The number of positive strains for each virulence factor and corresponding *p*-values are summarised in Table 4.

Discussion

Several studies demonstrated that MRSA infections in cancer patients are serious clinical conditions, with severe complications and high mortality rates [17, 18]. Since little is known about the characteristics of MRSA strains among hospitalised cancer patients in Saudi Arabia, it was considered important to elucidate the phenotypic and genotypic characteristics of local MRSA clones for the efficient management of infection in cancer patients.

In the current study, 120 MRSA isolates were obtained from cancer and non-cancer patients.

The *pvl* gene was not detected in any of the isolates from both groups. The *pvl* gene is considered as a stable genetic marker for community-associated MRSA (CA-MRSA) [19], hence, the absence of this gene is not surprising because all samples were collected from hospitalised patients and no community isolates were included in this study.

Table 3 Sequence types (STs) detected among MRSA isolates

eBURST CC	ST	Allelic profile	Cancer, *n* (%)	Non-cancer, *n* (%)	*p*-Value
Group 1	ST239	2-3-1-1-4-4-3	35 (58.3)	25 (41.7)	0.068
	ST08	3-3-1-1-4-4-3	3 (5)	4 (6.7)	0.697
	ST241	2-3-1-1-4-4-30	–	3 (5)	0.079
Group 2	ST22	7-6-1-5-8-8-6	6 (10)	4 (6.7)	0.509
	ST217	7-6-1-5-8-5-6	–	2 (3.3)	0.154
Singletons	ST80	3-3-1-14-11-4-10	13 (21.7)	12 (20)	0.822
	ST71	18-1-1-1-1-5-3	–	3 (5)	0.079
	ST30	2-2-2-2-26-3-2	–	3 (5)	0.079
	ST88	22-1-14-23-12-4-31	2 (3.3)	4 (6.7)	0.402
	ST182	18-18-6-2-13-15-18	1 (1.7)	–	0.315

The isolates were not only β-lactam resistant, but were often resistant to a range of other classes of antibiotics. This phenomenon is usually associated with HA-MRSA, which contrasts with the situation in CA-MRSA [20, 21]. A significant number of isolates showed resistance to antibiotics (other than β-lactams) that are frequently used in the treatment of MRSA infection; 68.3 % and 50 % (p=0.041) of strains from cancer and non-cancer patients were multi-resistant to three or more classes of antibiotics, including sulphonamides, lincosamides, macrolides, fusidic acid and ansamycins, with different resistance profiles. Nine and seven antibiotic resistance profiles were observed among the MRSA isolates from cancer and non-cancer patients, respectively.

Table 4 Adhesin and toxin gene profiles of MRSA isolates illustrated by numbers and *p*-values

Gene	Cancer (n=60)	Non-cancer (n=60)	*p*-Value*
fnbA	60	60	1
fnbB	25	36	0.068
clfA	60	60	1
clfB	60	60	1
fib	58	55	0.243
eno	60	60	1
cna	33	35	0.713
bbp	0	0	1
ebps	0	0	1
sea	9	3	0.068
seb	19	1	**0.0001**
sec	6	1	0.51
sed	0	0	1
see	0	0	1
sei	35	30	0.360
seh	16	3	**0.001**
seg	16	3	**0.001**
sej	0	0	1
eta, b	0	0	1
tst	9	0	**0.008**

*p-values <0.05 are statistically significant

The high level of multiple-drug resistance shown by the MRSA isolates from cancer compared to non-cancer patients in this study is of great concern (p=0.041). All the MRSA isolates showed resistance to at least two antibiotic classes, indicating the presence of strong selective pressure from antibiotic usage in this group. These differences in the antimicrobial resistance profiles of isolates from cancer versus non-cancer patients could be due to the differing pathologies of patients and the treatment regimens used. Patients with malignancy are usually associated with increased length of hospitalisation more than non-cancer patients, and are usually given antibiotics as part of the standard treatment. This supports the postulation that cancer patients may act as reservoirs of resistant bacteria. The development of simultaneous resistance to multiple antibiotic agents may contribute to the impediment of MRSA treatment.

Currently, a wide variety of genotype-based typing methods are available for classifying MRSA isolates. SCC*mec* is an important typing target. All MRSA strains in this study belonged to SCC*mec* type III, which was previously identified as the predominant type in Saudi Arabia [22].

No significant correlation was observed in the distribution of *spa* and MLST types among cancer and non-cancer patients (p>0.05). As the distribution of MRSA genotypes has already demonstrated a distinct demarcation according to the geographic origin of isolates [22], these isolates are most likely to reflect the differences in their epidemiology, as the strains were collected from the different departments of major government referral hospitals for patients from all cities in Saudi Arabia.

In comparison, *spa* and MLST typing showed good concordance with each other. However, *spa* typing has a very good predictive power, ease of use, speed and global interpretation [23].

The prevalence of adhesin genes varied slightly among MRSA isolates from cancer and non-cancer patients, but

such variations were not statistically significant. This finding is in agreement with a previous report which indicated that the prevalence of adhesin genes was consistent, regardless of the clinical source [24]. It was shown, in various studies, that the *cna* gene was an important virulence factor in the pathogenesis of septic arthritis and bone tissue infections. Peacock et al. [25] compared the presence of virulence genes in *S. aureus* isolates from healthy human blood donors and from patients with invasive disease. They noticed that the *cna* gene was significantly more common in invasive isolates. In our study, this could not be confirmed or rejected, as all our isolates were from infectious origin.

It is known that *S. aureus* strains produce one or more specific staphylococcal enterotoxins and toxic shock syndrome toxin. However, relatively little is known about the distribution of virulence factors among MRSA isolates. The present study is, to our knowledge, the first study showing a comprehensive comparison of the toxin genotypes of MRSA, as there are no available published reports on the presence of these genes in Saudi Arabian MRSA isolates.

These *sei* and *seg* genes belong to the same enterotoxin gene cluster (*egc*) and this parallel incidence has been described in previously published reports [26]. In contrast, isolates that were positive for *sei* were not always positive for *seg*, even though these genes are part of the *egc* gene cluster, confirming some variability in this cluster [27]. The *tst* gene was always detected in combination with other genes. The *tst* gene is considered to co-exist with the enterotoxin gene cluster (*egc*), which includes *seg* and *sei* [2, 28]. However, among nine isolates harbouring the *tst* gene, six isolates (72.2 %) were positive for at least one enterotoxin gene in our study.

The frequency of the *seb*, *seh*, *seg* and *tst* genes was significantly higher ($p<0.05$) in MRSA strains isolated from cancer patients. Although these findings may indicate the potential pathogenic effects of these isolates in cancer patients, it is important to recognise that the presence of the enterotoxin genes in *S. aureus* isolates does not necessarily indicate the ability of these isolates to produce intact and biologically active toxin or to produce sufficient toxin to induce disease [29]. A study by Lindsay et al. [30] also demonstrated this non-association of specific genes or combinations of genes with invasive isolates, and there were no consistent differences in the gene content that could be used to distinguish between invasive and carriage isolates. Hence, further study to investigate the toxin gene expression levels of MRSA in cancer patients is suggested.

One aim of our study was to investigate the presence of virulence factors, alone or in combination, in correlation to genotypes and resistance profiles. Isolates of each ST were grouped according to the virulence factors, genotypes and resistance profiles determinants which they harboured. The antibiotic-resistant isolates showed the presence of various virulence factors, including adhesin and toxin genes. Similarly, the antibiotic-susceptible isolates were also found to reveal these pathogenic factor genes. The presence or absence of these genes indicated non-significant correlations to antibiotic-resistant or antibiotic-susceptible aspects.

The identification of strains with variable toxin gene profiles belonging to different clones indicates that enterotoxin genes are not strictly linked, and a multitude of combinations among themselves and other enterotoxins may occur in bacterial populations [28]. The different distributions of toxin genes among different clonal groups and genomic backgrounds are expected, as most of the enterotoxin genes are frequently carried by potentially mobile elements, such as plasmids, phages and pathogenicity islands [2, 31].

In conclusion, this study demonstrated that, although all MRSA strains studied from cancer and non-cancer patients possessed several virulence determinants with similar molecular backgrounds, the isolates from cancer patients were more often multiple resistant for antibiotic agents and the presence of toxin genes was also significantly higher in cancer patients. These findings may suggest that MRSA is a serious clinical pathogen, especially among cancer patients. Hence, the attempts to control the spread of MRSA in hospitals should continue. Hospitals should also develop policies to restrict the use of antibiotics and establish monitoring systems with at least the *spa* typing technique for the rapid identification of epidemics and determination of circulating and new emerging clones, which will lead to improved prevention and treatment strategies.

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Conflict of interest The authors declare that they have no conflicts of interest.

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