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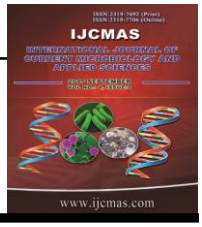

Original Research Article

Hospital-Acquired Methicillin Resistant *Staphylococcus aureus*: Analysis of mec A Gene and Staphylococcal Cassette Chromosome

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Keywords

Methicillin resistant *Staphylococcus aureus*, *Staphylococcus* cassette chromosome, *mecA* gene, Penicillin-binding protein, Panton-Valentine Leukocidin

A B S T R A C T

Staphylococcus cassette chromosome (SCC)is a variable genetic element that contains the *mecA* and is considered the most reliable method for detection of *Methicillin resistant Staphylococcus aureus* (MRSA). The aim of this study was to characterize hospital acquired MRSA (HA-MRSA), both phenotypically and genotypically. Fifty staphylococci strains were isolated from hospitalized patients, from which38 (76%) were mannitol fermenters. Twenty strains (40%) were resistant to both oxacillin and cefoxitin, however, 2 (4%) isolates were resistant to oxacillin only and 7 (14%) were resistant to cefoxitin only. The 29 resistant strains were diagnosed as MRSA by detection of *mecA* gene using real time PCR. They were allpenicillin-binding protein2a producers. Among these isolates,26 (89.66%) were typable by using Zhang set of primers. Only 3 (10.34%) of the 29 isolates were Panton-Valentine Leukocidin positive, from which 2 were *SCCmec* type IV and V, while the third remained untypable. The combination of phenotypic characteristics in conjunction with antibiograms are useful to a certain extent, but further work is required to find a reliable marker to facilitate the recognition ofHA-MRSA.

Introduction

Staphylococcus aureus (*S.aureus)* continues to be a serious health problem worldwide due to its intrinsic nature of virulence, its ability to cause a wide array of infections, and its capacity to develop resistance to a number of antibiotics with the ability for clonal dissemination, through both mutation and acquisition of exogenous genes (Deurenberg and Stobberingh,2008).

S. aureus can cause potentially serious healthcare associated infections. In 1959, methicillin was introduced to overcome the resistance problem. However, the first

MRSA was isolated shortly after the introduction of methicillin (Lee *et al.*, 2011).

Virulence factors are encoded by phages, plasmids, pathogencity islands and the *SCC*. Increased resistance for antibiotics is encoded by a transposon (Tn 1546) inserted into a conjugated plasmid that has also encoded resistance to disinfectants (Chambers and Deleo, 2009, Lee *et al.*, 2011).

The resistance to methicillin is not due to β-lactamase production but to the expression of an additional penicillin-binding protein (PBP_{2a}), acquired from other species, which was resistant to the action of the antibiotic, and this protein is coded for by a mobile genetic element termed the methicillin-resistant gene (*mecA*) (My *et al.*, 2011). PBP_{2a} has low affinity to methicillin rendering the bacteria that produce it; resistant to all beta-lactam antibiotics(Kim *et al.*, 2012). In recent years, the gene has continued to progress so that many MRSA strains are currently resistant to several antibiotics such as penicillin, oxacillin, and amoxicillin(Chambers and Deleo, 2009).

The rates of MRSA infections in hospitals, as well as thecommunity, have continued to rise.The differentiation between the typical HA-MRSA and community acquired MRSA (CA-MRSA) strainsbased on epidemiologic definitions has become difficult(Yamamoto *et al.*, 2010, Mediavilla 2012).

SCCmec is a variable genetic element that contains the methicillin resistance determinant, *mecA*. *SCCmec* typing is one of the most important molecular tools available for distinction between HA-MRSA andCA-MRSA (Turlej*et al.*, 2011).

HA-MRSA strains are typically associated with one of three types I–III, which carry additional antibiotic resistance genes. In contrast, CA-MRSA has almost exclusively been associated with type IV. More recently, type V *SCCmec* is described to be harboured by *S. aureus* strains which behave similarly to type IV strains, causing typical CA-MRSA infections. (Turlej *et al.*, 2011, Shanshuang *et al.*, 2011)

In 1932, Panton and Valentine described Panton-Va lentine Leukocidin (PVL), a leukocidintoxin as a virulence factor that form pores in the membrane of host defense cells by the synergistic action of two secretory proteins, designated LukS-PV and LukF-PV (Tristan *et al.*, 2007).

These proteins are encoded by two cotranscribed genes of a prophage integrated in the *S.aureus* chromosome and are secreted before they assemble into a pore-forming polymorphonuclear leukocytes membranes, leading to their lysis (Tristan *et al.*, 2007, Shallcross*et al.*, 2012).

PVL is mostly associated with CA-MRSA infections and is distinguishable from HA-MRSA by nonmultidrug resistance and the carriage of SCCmectype IV. (12) PVL is widely associated with the presence of SCCmec IV and sporadically with SCCmec V but not SCCmec types I, II or III (David and Daum, 2010).

This study aimed to characterize HA-MRSA both phenotypically and genotypically.

Material and Methods

A total of 50 staphylococci strains were isolated from hospitalized patients whom had developed infections 48 hours after their admission. Specimens included pus from wounds, catheters, blood, sputum and vaginal swabs.

All samples were inoculated on blood agar and MacConkey agar (Oxoid Ltd, Wade Road, Basingstoke, Hampshire, UK) plates. Staphylococcal isolates were identified by their colonial appearance. Colonies suspected as staphylococci were Gram stained and tested for catalase, oxidase, slide coagulase and tube coagulase tests (Baird, 1996).

Susceptibility of staphylococci was determined by the disc diffusion method that included oxacillin and cefoxitin discs. (Clinical and Laboratory Standards Institute, 2009).

PBP2a Latex Agglutination Test (OXOID®) was performed according to the manufacturers' instructions to detect the presence of PBP2a responsible for methicillin resistance. Agglutination seen with the test but not the control, within 3 minutes was considered PBP_{2a} positive (Hussain *et al.,* 2000).

Genotypic identification of MRSA was carried out by detecting *mecA* gene encoding the low affinity PBP2a.Molecular typing of MRSA was carried out using Zhang *et al.,* (2005) scheme targeting *mec* gene (Class A and B), *ccr* gene complexes and individual *SCC*mec types and subtypes using real time PCR by SYBR Green1 technique. Real time PCR was used to determine the occurrence of PVL as a virulence factor (Jones *et al.,* 2007, Enany*et al.,* 2010).

Staphylococcal DNA Extraction

Staphylococcal isolates were subcultured overnight at 37°C on blood agar media. Few colonies were emulsified in 200 µl sterile distilled water to produce a heavy suspension. The bacterial suspension was heated at 100°C for 15 min. The suspension was then centrifuged at 14, 000 rpm for 5 min.

Reagents

SYBR Green universal PCR master mix 2-fold was purchased from (Fermentas life sciences®). All Primers were purchased from metabion international AG and were reconstituted with sterile distilled water to obtain a concentrate of 100 picomoles/µl.

Protocol of amplification

Each PCR tube contained the following:

12.5 µl SYBRGreen universal PCR master mix 2-fold**,** 0.5 µl forward and reverse primer**,** 5 µl DNA extract**,** and 6.5 µl PCR grade water bringing the reaction volume to 25µl.A negative control was prepared by the addition of the same contents to the tube with water instead of the extract.

The tubes were placed in the thermal cycler for amplification. The thermal profile included 1 Cycle of activation at 95 ºC for10 min., followed by 35 Cycles of denaturation at 95 ºC for15 sec, annealing at55 ºC for 30 sec., and extension at 72 ºC for 30 sec. This was followed by the melting curve analysis of 1 Cycleat 95 ºC for1 min., 55ºC for 30 sec., and 95 ºC for 30 sec.

Amplification was done for SCC*mec* typing or detection of *PVL* gene using the same protocol and thermal profile as for *mecA* amplification except that the annealing temperature varied according to the amplified gene. Annealing temperature was50ºCfor Zhang *et al* primers amplifying SCC*mec*type II, III, IVb, IVc class A and B 53ºC for SCC*mec* type IVa and IVd, 55ºC for SCC*mec*type V and 60ºC for *PVL*gene.

Results and Discussion

The 50 (94.34%) staphylococci isolates were positive for the both the clumping factor

(detected by the slide coagulase test) and free coagulase (detected by the tube coagulase test) and were thereby diagnosed as *S.aureus.*

The presence of *mecA* genes detected by real time PCR was considered to be the gold standard in the diagnosis MRSA (figs.1 and 2). Out of the 50 *S. aureus* isolated from hospitalized patients, 29 (58%) isolates were positive for *mecA* gene and were PBP_{2a}producers; hence were diagnosed as MRSA; among which 27 (93.01%) were resistant to cefoxitin while only22 (75.86%) isolates were found to be resistant to oxacillin. The antibiotic sensitivity of HA-MRSA ranged from 58.62% for co-trimoxazole, erythromycin or tetracycline to 96.55% for rifampicin.

Molecular typing:

Regarding the 29 HA-MRSA strainsthat were initially tested with SCC*mec* class A and class B primers (Zhang *et al.,* 2005) ; 11 (37.93%) strains were SCC*mec* class A positive, 6 (20.69%) strains were SCC*mec* class B positive, while, 12 (41.38%) strains couldnot be classified as class A nor B.

When testing the 11 class A positive cases with SCC*mec ccr*2 and SCC*mec ccr*3 primers (Zhang *et al.,* 2005), only 4 cases were positive: 2 cases (18.18%) were positive with SCC*mec ccr*2 primers, and were thereby considered as SCC*mec* type II. The 2 (18.18%) other cases were positive with SCC*mec ccr*3 primers and were considered as SCC*mec* type III. The 7 (63.64%) remaining class A positive cases were negative for both SCC*mec ccr*2 and *ccr*3 primers.

When testing the 6 class B positive cases withSCC*mec ccr2* and SCC*mec ccr1* primers, only 3 cases (50%) were positive, 2 cases (33.33%) were positive withSCC*mec ccr2* primers andwerethereby considered as SCC*mec* type IV. The other case (16.67%) was positive withSCC*mec ccr1* primers and was considered as SCC*mec* type I.

The remaining 22 untypable HA-MRSA strains (10 class A or B positive, *ccr* negative strains and 12 class A and B negative strains) were tested with an individualset of Zhang *et al.,* (2005) primers forSCC*mec*type I, SCC*mec*type II, SCC*mec*type III and SCC*mec*type V.

Out of the 10 class A or B positive*ccr* negative strains, 7 cases (70%) were SCC*mec*type I and 3 (30%) cases were SCC*mec*type III.Out of the 12 class A and B negative strains, 6 cases (50%) were SCC*mec*type V, 3 cases (25%) were SCC*mec*type I and 3 cases (25%) remained untypable.

Fig. 3. shows the distribution of the 29HA-MRSA, where 26 (89.66%) were typable by Zhang *et al.,* (2005) ; set of primers) as follows: 11 (37.93%) wereSCC*mec* type I, 2 (6.90%) were type II, 5 (17.24%) were type III 2 (6.90%) were type IVa and 6 (20.69%) were type Vand 3 (33.34%) remained untypable.

S. aureus has a remarkable ability to develop antibiotic resistance. MRSA first emerged as a healthcare associated pathogen in the1960s.Since the 1980s, the emergence of MRSA has become widespread in hospitals and long-term care facilities, accounting for numerous healthcare associated infections. It is a global public health problem and represents the most commonly identified antibiotic-resistant pathogen (Ray*et al.,* 2011and Tong *et al.,* 2015).

A total of 50 *S. aureus* isolates were collected from infected wounds of

hospitalized patients in the Medical Research Institute, Alexandria University. They were identified as Gram-positive, catalase positive, oxidase negative and coagulase positive cocci.

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by *S. aureus*. Identification of the *mec*A gene is the most reliable method of detecting MRSA isolates (Turlej *et al.*, 2011, Shanshuang *et al.*, 2011).

In the present study the presence of *mecA* gene was detected by real time PCR using the SYBR Green 1 dye. This method obviates the need for target-specific fluorescent probes, reducing assay setup and running cost, but its specificity is determined entirely by the primer sequence. However, melting curve analysis, depending on GC content and the size of the amplicon, was carried out to identify the amplification product and to determine its purity and specificity. Melting curve analysis of the *mecA* positive strains gave excellent results regarding specificity, where the position of the peaks were identical for all strains.

Salimnia and Brown (2005) evaluated oxacillin and cefoxitin disc diffusion utilizing *mec*A as the gold standard, the category agreement for oxacillin disc was 93.3% and for cefoxitin disc 99.7%.

Velasco *et al.*, (2005) found that 5.9% out of their strains that were positive for the mecA gene yielded false negative results with the oxacillin disc diffusion. They explained the lower sensitivity by the absence of, or reduced expression of, the *mecA*-encoded protein, PBP_{2a}; suggesting that antibiotics, able to induce expression of methicillin resistance such as cefoxitin, are the most appropriate for detecting MRSA isolates, as all mecA-positive isolates were detected with the cefoxitin disc with a sensitivity of 100%. (Gupta*et al.*, 2009)

Out of the 50 *S.aureus* strains isolated from hospitalized patients 29 (58%) were positive for the *mecA* gene and were PBP_{2a} producers, 27 (93.01%) out of them were resistant to cefoxitin disc while only 22 (75.86%) isolates were resistant to oxacillin, discconfirming that identification of MRSA is more accurate by either directly detecting the gene encoding the methicillin resistance determinant (*mecA*) or its product, penicillin-binding protein 2a (PBP_{2a}).

In the present study, out of the 29 HA-MRSA 14 (48.28%) isolates were found to be multidrug resistant. These isolates were resistant to 2 or more antibiotic classes including quinolones, co-trimoxazole, erythromycin and tetracycline. A 41.38% were found resistant to co-trimoxazole, 31.03% to quinolones and only 3.45% to rifampicin. All isolates were sensitive to vancomycin.

Zhang *et al.*, (2005) typing scheme targets the individual regions of the classes of the mec complex (IS431-*mecA*, IS1272-*mecA*, and *mecI*-*mecRI*). Four primers were used to identify class A and B of the *mec* gene complex, the allotypes of the *ccr*complex were identified using 6 primers. SCCmec types I to V were identified by the combination of the *mec* classes and *ccr* allotypes.

Out of the 29 HA-MRSA, 11 strains were positive for class A, 6 strains were positive for class B and 12 strains were negative for both class A and B. By *ccr*typing, only 4 strains out of the 11 class A positive strains were positive for *ccr* genes: 2 strains were positive for *ccr*2 and were classified as SCC*mec* type II and 2 strains were positive

for *ccr*3 and were classified as SCC*mec* type III. The remaining 7 class A positive strains were *ccr* negative & thereby couldn't be assigned to any SCC*mec* type. Also, only 3 strains out of the 6 class B positive strains were positive for *ccr* genes: 2 strains were positive for *ccr*2 and were classified as SCC*mec* type IV and 1 strain was positive for *ccr*1 and was classified as SCC*mec* type I.The remaining 3 class B positive strains were*ccr* negative & thereby couldn't be assigned to any SCC*mec* type.

Table.1 Initial set of primers were used to identify mecA, SCCmec and PVL gene

Primer Type	Primers	Oligonucleotide sequence (5'-3')	Ampl-icon size (bp)	Specificity
mec A (Killgore *et al.*, 2000)	M1-F M2-R	885-TGGCTATCGTGTCACAATCG-904 1194-CTGGAACTTGTTGAGCAGAG-1175	310	mecA
Zhang *et al.*, (2005)	Type I-F Type I-R	GCTTTAAAGAGTGTCGTTACAGG GTTCTCTCATAGTATGACGTCC	613	SCCmec I
	Type II-F Type II-R	CGTTGAAGATGATGAAGCG CGAAATCAATGGTTAATGGACC	398	SCC*mec*II
	Type III-F Type III-R	CCATATTGTGTACGATGCG CCTTAGTTGTCGTAACAGATCG	280	SCC*mec*III
	Type IVa-F TypeIVa-R	GCCTTATTCGAAGAAACCG CTACTCTTCTGAAAAGCGTCG	776	SCCmec Iva
	Type IVb-F TypeIVb-R	TCTGGAATTACTTCAGCTGC AAACAATATTGCTCTCCCTC	493	SCCmecIVb
	Type IVc-F TypeIVc-R	ACAATATTTGTATTATCGGAGAGC TTGGTATGAGGTATTGCTGG	200	SCCmecIVc
	Type IVd-F Type Vd-R6	CTCAAAATACGGACCCCAATACA TGCTCCAGTAATTGCTAAAG	881	SCCmecIVd
	Type V-F Type V-R	GAACATTGTTACTTAAATGAGCGTG AAAGTTGTACCCTTGACACC	325	SCC*mec*V
	mecI- F mecI-R	CCCTTTTTATACAATCTCGTTATATCA TCTGCAGAATGGG	146	Class A mec
	IS1272- FmecR1-R	TATTTTTGGGTTTCACTCGGCTCCACG TTAATTCCATTAATACC	1, 305	Class B mec
PVL (Jones *et al.*, 2007, Enany *et al.*, 2010)	luk-PV-1 luk-PV-2	ATCATTAGGTAAAATGTCTGGACATG ATCCAGCATCAASTGTATTGGATAGC AAAAGC	433	PVL

Fig.1 Shows amplification plots (fluorescence against cycle number) of the mecApositive strains with different Ct.

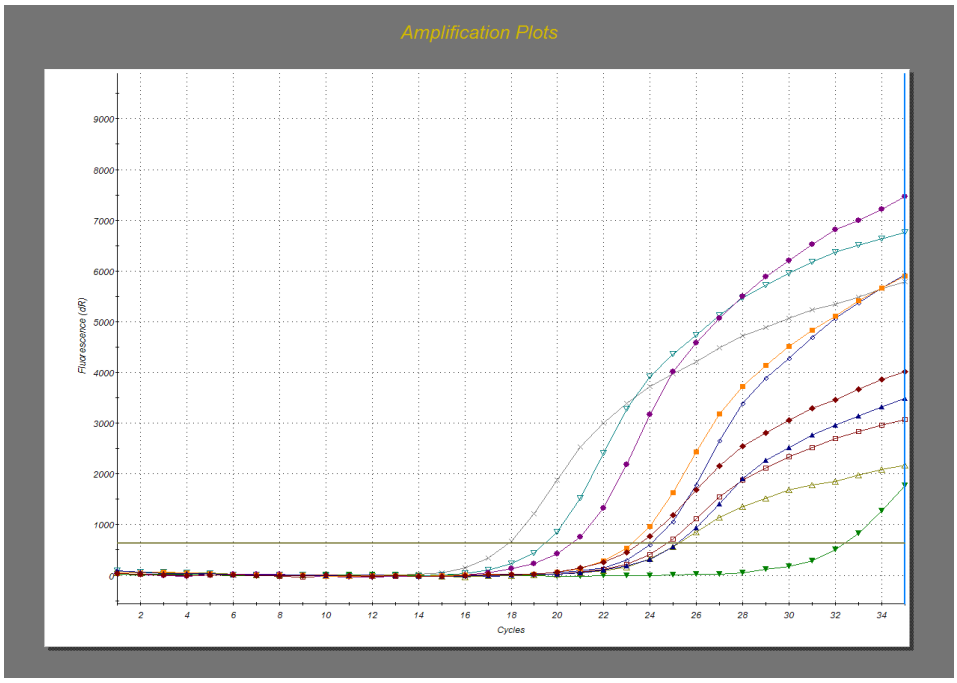


Fig.2 Melting curve analysis of the *mecA* positive strains to identify the amplification product and to determine its purity and specificity

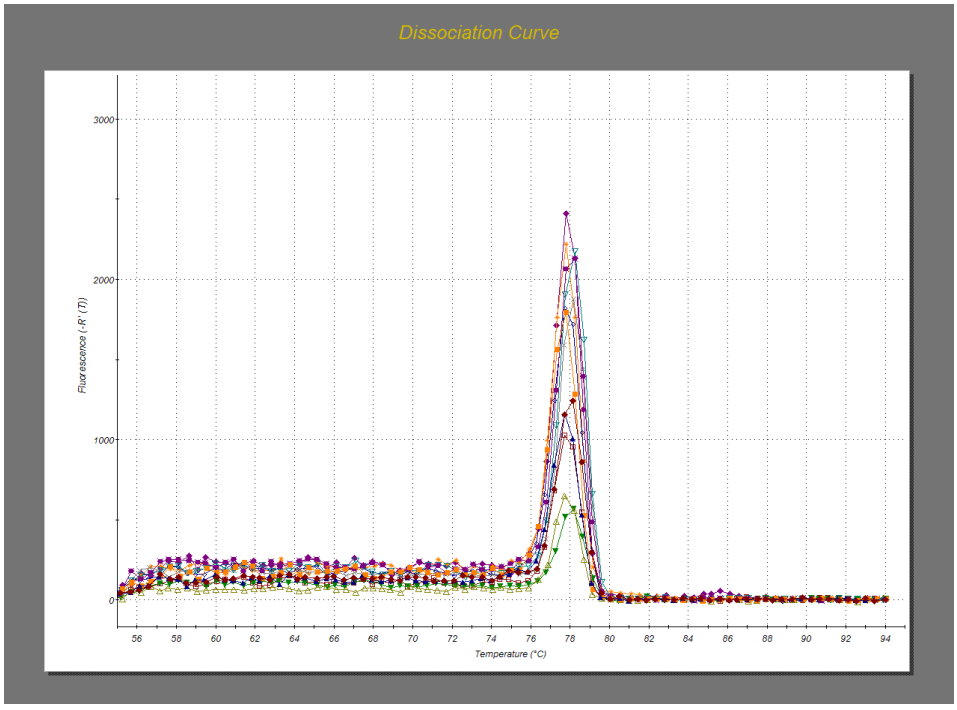
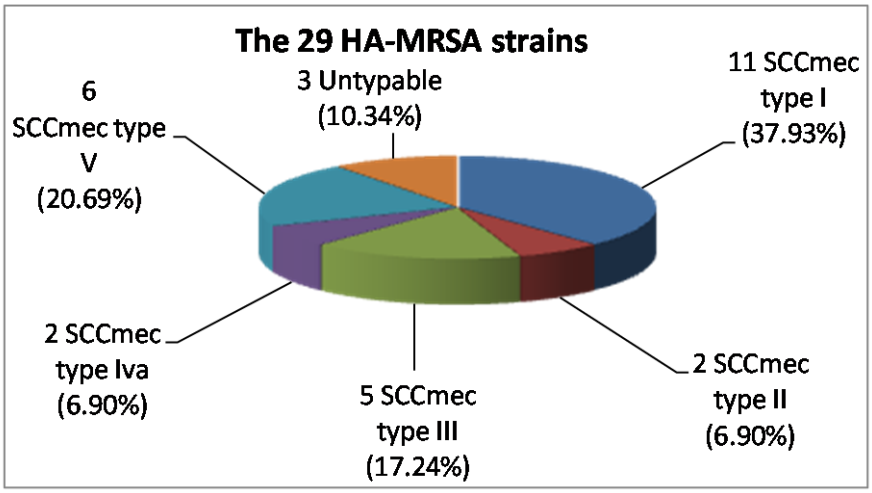


Fig.3 Distribution of different SCCmec types among the 29 MRSA isolates



By this method of typing, out of 29 HA-MRSA, only 7 (24.14%) strains were typable: SCC*mec* type I [1 isolate (3.45%)], SCC*mec* type II [2 isolates (6.90%)], SCC*mec* type III [2 isolates (6.90%)] and SCC*mec* type IV[2 isolates (6.90%)].

Zhang *et al,* (2005) designed 8 sets of specific primers based on analyses and alignments of the MSSA and MRSA genomes and variable SCC*mec*type and subtype sequences in the GenBank database.

In the present study, these sets of primers were used to type the 22 strains which were not typable otherwise: class A or B positive, *ccr* negative (10 strains) and class A and B negative (12 strains) :19 (86.36%) out of these 22 strains could be classified by this method. 10 (45.45%) strains were SCC*mec* type I, 3 (13.64%) strains were SCC*mec* type III, 6 (27.27%) strains were SCC*mec* type V and 3 (13.64%) strains remained untypable.

By using Zhang *et al.,* (2005) scheme, 26 (89.66%) out of the 29 HA-MRSA weretypable. SCC*mec* type I: 11 (37.93%), type II: 2 (6.90%), type III: 5 (17.24%), type IVa: 2 (6.90%) and type V: 6 (20.69%), while 3 (10.34%) remained untypable.

Type V SCC*mec* is structurally similar to type IV SCC*mec*, in that it contains *mec*Aas the only gene encoding antibiotic resistance. Its size (28 kb) was also comparable to that of type IV SCC*mec* (21 Kb) and was much smaller than those of the type I to III SCC*mec* elements (34 to 67 kb), (Valsesia*et al.*, 2015).

There has been much interest in *PVL*, due to its involvement in severe disease among children and young adults with no known exposure to healthcare establishments. CA-MRSA has been reported to carry the loci for *PVL* in high frequency, and to be associated with the SCC*mec*type IV. (Katopodis*et al.*, 2010, Sobhy N*et al.*, 2012., Valsesia*et al.*, 2015)

However, only 12%out of 108 CA-MRSA isolated in Finland (Fang*et al.,* 2008) contained the *PVL* gene, moreover 6.7% of isolates from Irish patients (Rossney*etal.*, 2011) with an epidemiological history of CA-MRSA carried *PVL* genes.Similarly, 78% of CA-MRSA isolates, referred to a

central reference facility for investigation of the carriage of *PVL*, were negative.

However, 3 (10.34%) out of the 29 HA-MRSA isolates were *PVL* positive and 2 (6.9%) were SCC*mec*type IVa. These findings confirm that molecular typing forHA andCA-MRSA differentiationcannot be used as a sole tool.

In this study, the combination of *PVL*, SCC*mec*type IVa and a few untypable strains were not sufficient to identify all presumably HA-MRSA strains isolated from the hospital.

The combination of phenotypic characteristics in conjunction with antibiograms are useful to a certain extent, but further work is required to find a reliable marker or combinations of markers to facilitate the recognition of HA-MRSA.

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