

Full Length Research Paper

## Phenotypic and genotypic characterization of methicillin and vancomycin resistant staphylococci

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One hundred (100) staphylococci were isolated from several Egyptian hospitals and laboratories which include 85 clinical isolates and 15 from hospital surroundings. The isolates were identified by conventional and molecular techniques. Fifty (50) isolates were identified as *Staphylococcus aureus* (SA), 40 were *Staphylococcus epidermidis* (SE) while 10 were identified as *Staphylococcus* species (SS). Upon testing the resistance to methicillin and vancomycin, it was found that resistance is dominant in isolates from clinical samples than those from the surrounding surfaces. Moreover, the resistance to methicillin was higher than that to vancomycin. Multiplex polymerase chain reaction was carried out to characterize the staphylococci-specific region of 16S rRNA gene, *mecA* gene associated with methicillin resistance and the virulence marker-associated genes Pantone-Valentine leukocidin (PVL) *lukS/F-PV* genes which are responsible for leukocyte destruction and tissue necrosis. All the methicillin resistant staphylococci (MRS) were found to be *mecA*<sup>+</sup> while only five MRS carried *lukS/F-PV* genes. On the other hand, 30% of the methicillin sensitive staphylococci (MSS) were found to harbor the *mecA* gene while lacking the PVL. The results highlight the important role of horizontal gene transfer of virulence genes between staphylococci. In addition, this study indicates that the use of multiplex PCR is not sufficient for antibiotic susceptibility prediction and thus the simultaneous use of conventional and multiplex PCR technique is required for the identification of staphylococci and determination of their antibiotic susceptibility.

**Key words:** Methicillin resistance, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *mecA*, Pantone-Valentine leukocidin (PVL).

### INTRODUCTION

Staphylococci are opportunistic human pathogen capable of causing a wide variety of diseases. The severity of a staphylococcal infection and its response to antibiotic treatment is dictated by the specific suite of virulence and antibiotic resistance associated genes (Peacock et al., 2002). Staphylococci impose challenge to clinicians, not only because of vancomycin and methicillin resistance, but also because of resistance to many other antibiotics (Levy and Marshall, 2004). Detection of methicillin resistance can be difficult due to the presence of two subpopulations, one susceptible and the other resistant that may coexist within a culture of staphylococci. This

phenomenon is termed heteroresistance. Results using conventional phenotypic assay may be given after 48 h or more. In case of severe disease, the early detection of MRSA is essential. Thus detection of *mecA* gene using PCR is considered to be the best method (Prere et al., 2006).

Hospital associated methicillin resistant *Staphylococcus aureus* (HA-MRSA) acquired an integrated sequence into their genome (21-67-kb mobile genetic element), termed staphylococcal cassette chromo-some *mec* (SCC*mec*), which harbours the methicillin resistance gene *mecA* (Ito et al., 2001, 2004). The SCC*mec* chromosome contains

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*mecA* gene and two regulatory genes *mecI* and *mecR1* which constitute *mec* complex that play an important role in the regulation of the *mecA* gene (Archer and Bosilevac, 2001; Berger-Bachi and Rohrer, 2002). Community-associated MRSA strains carry the Panton-Valentine leukocidin (PVL) virulence genes possessing a small mobile staphylo-coccal cassette chromosome *mec* (SCC*mec*) type IV or V genetic element which harbors the methicillin resistance (*mecA*) gene and which is more easily transferred to other strains of *S. aureus* than the larger SCC*mec* types (types I to III) that are prevalent in hospital associated MRSA strains (Vandenesch et al., 2003; Zhang et al., 2004).

McClure et al. (2006) developed a multiplex PCR to detect the staphylococci specific region of 16SrRNA gene along with virulence genes such as Panton-Valentine leukocidin (PVL) *lukS/F-PV* genes; which is responsible for leukocyte destruction and tissue necrosis and *mecA* gene associated with methicillin resistance. Concordance between phenotypic and genotypic characteristics was recorded.

The aim of this work was to determine whether the use of multiplex PCR amplifying these three genes would suffice the identification and prediction of virulence and the antibiotic susceptibility pattern of isolated staphylococci.

## MATERIALS AND METHODS

### Bacterial isolates

A total of 100 clinical and hospital surroundings samples were collected from five different hospitals in Cairo. Samples from blood, urine, sputum, pus, throat, wound swabs, soil and patient beds were collected.

Samples were first collected on nutrient agar plates, purified and then sub-cultured on plates of blood agar, mannitol salt and Baird-Parker agar medium (Oxoid) using the streak plate method. The plates were incubated at 37°C for 24 - 48 h. Preliminary identification was carried out as recommended by Mahon and Manusekis (1995), Chapin and Lauderdale (2003) and Todar (2005). Gram stain, catalase and coagulase production were carried out to identify staphylococcal isolates (Cheesbrough, 1984; Koneman, 1992).

### Antibiotic susceptibility test

Muller Hinton plates were inoculated with 0.5 McFarland standard inocula then different antibiotic disks were placed on the surface of the agar plates (methicillin 6 µg, ampicillin 10 µg, vancomycin 10 µg, linezolid 30 µg, clindamycin 2 µg, ciprofloxacin 10 µg, tobramycin 10 µg, erythromycin 15 µg, doxycycline 30 µg and cefoperazone 75 µg). The antibiotic susceptibility test was carried out according to Kirby-Bauer disk diffusion susceptibility test protocol (Bauer et al., 1966) and the inhibition zones were measured as recommended by NCCLS (1997).

### Determination of minimal inhibitory concentration (MIC)

Minimal inhibitory concentration of methicillin and vancomycin was

tested according to Washington and Wood (1995) and NCCLS (1997).

Staphylococci are considered methicillin resistant (MRSA) if their MIC for oxacillin is  $\geq 4$  µg/ml and they are considered vancomycin resistant (VRSA) if their MIC for vancomycin  $\geq 16$  µg/ml (NCCLS, 1997).

## Polymerase chain reaction

Genomic DNA was extracted from the bacterial isolates according to Sambrook et al. (2001). PCR were performed with three primer pairs. The first pair is *mecA1* (5'-GTAGAAATGACTGAACGTCCGATAA-3') and *mecA2* (5'-CCAATTCCACATTGTTTCGGTCTAA-3') corresponding to the *mecA* gene which encodes the unique penicillin-binding protein associated with oxacillin resistance in staphylococci (Zhang et al., 2004). The second pair is Staph756F (5'-AACTCTGTTATTAGGGAAGAACA-3') and Staph750R (5'-CCACCTTCTCCGGTTTGTACC-3') corresponding to regions of 16S rRNA genes that are unique to staphylococci (Zhang et al., 2004). The third pair is *LukPV-1* (5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3') and *LukPV-2* (5'-CATCAAGTGTATTGGATAGCAAAGC-3') corresponding to the virulence marker (PVL) *lukS-PV* and *lukF-PV* genes that encode for tissue necrosis in staphylococcal infection (Lina et al., 1999). The PCR amplification products of the three primer pairs *mecA*, 16S rRNA and PVL genes are 310, 756 and 433 bp, respectively.

A routine laboratory PCR method for the detection of *lukS-PV* genes and *mecA* gene have required the use of separate PCR programs to investigate the proper conditions for each gene (Van Hal et al., 2007). Multiplex PCR conditions were performed according to McClure et al. (2006) and Van Pelt-Verkuil et al. (2008). One micro liter of DNA was added to 30 µl final reaction volume containing 3 µl primers mix (10 pmol specific for 16 srRNA, *lukS-PV* and *mecA* genes), 3 µl Taq Buffer (10x), 1.8 µl Taq Enzyme (25 Mmol mgcl), 3 µl (2Mmol DNTase) and 13.7 µl nuclease-free distilled H<sub>2</sub>O. The PCR was performed using AB Applied Biosystems thermocycler 2720. The PCR protocol was one denaturation step at 95°C for 5 min, followed by 25 cycle of denaturation at 95°C for 1 min annealing at 57°C for 1 min and primer extension at 72°C for 1 min, post extension step at 72°C for 10 min. Amplified PCR products were recognized in 0.8% agarose gel stained with ethidium bromide.

## RESULTS

Eighty five (85) staphylococcal isolates were from clinical samples and 15 from hospital surfaces. Data were collected based on colony morphology, growth on blood agar media, mannitol salt agar media and Baird-Parker agar media, catalase and coagulase tests. Fifty isolates produced golden yellow colonies on mannitol salt agar medium and they were positive for catalase and coagulase. Thus, they were identified as *S. aureus* (SA). Forty isolates formed pink colonies on mannitol salt agar medium and were tested positive for catalase production but negative for coagulase and were identified as *Staphylococcus epidermidis* (SE). Ten isolates were atypical to either SA or SE. Therefore they were designated as *Staphylococcus* species (SS). The percentage of occurrence is shown in Table 1. The highest occurrence of staphylococci was found in wound infections

**Table 1.** Sources and numbers of staphylococcal isolates, *S. aureus* (SA), *S. epidermidis* (SE) and unidentified *Staphylococcus* sp. (SS).

Source	Total number (%)	No. of SS isolates	No. of SE isolates	No. of SA isolates
Floor	6	1	2	3
Lab bench	3	0	1	2
Patient bed	6	1	3	2
Blood	9	0	4	5
Urine	19	2	10	7
Sputum	7	0	2	5
Pus discharge	12	2	3	7
Wound swab	21	1	7	13
Throat swab	11	1	6	4
Ear swab	3	0	1	2
Cerebrospinal fluid (CSF)	1	1	0	0
Pleural fluid	1	1	0	0
Cerebral fluid	1	0	1	0
Total no	100	10	40	50

**Table 2.** Distribution of methicillin resistant staphylococci (MRS) and vancomycin resistant staphylococci (VRS) according to their minimal inhibitory concentration (MIC).

Resistance (%)	(SA) (n=50)		(SE) (n=40)		(SS) (n=10)	
	Sur (n=7)	Clin (n=43)	Sur (n=6)	Clin (n=34)	Sur (n=2)	Clin (n=8)
MRS (MIC $\geq$ 4 $\mu$ g/ml)	28.5%	53.5%	0%	41%	100%	100%
VRS (MIC $\geq$ 16 $\mu$ g/ml)	0%	25.5%	0%	32.5%	0%	25%

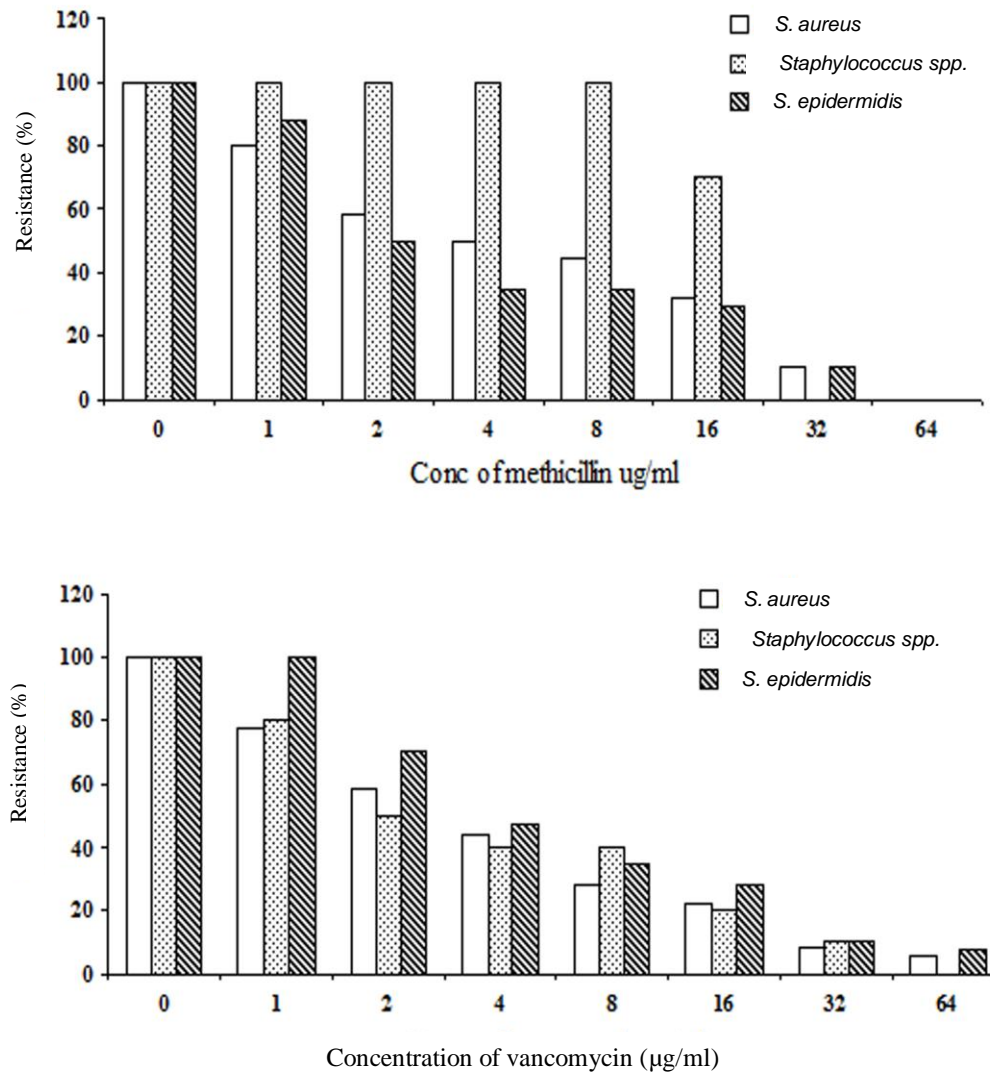
followed by urine samples. In addition, SA strains were more dominant in wound infections while (SE) strains were more dominant in urine samples.

Antibiotic susceptibility test showed multi-drug resistance to tested antibiotics with various extents. Forty seven percent of all tested staphylococci were resistant to methicillin, 31% were resistant to vancomycin, 52% to ampicillin, 40% to ciprofloxacin, tobramycin and erythromycin, 36% to doxacyclin, 33% to clindamycin and 7% were resistant to linezolid.

MIC of methicillin and vancomycin were measured for all the staphylococcal strains. Strains able to grow at concentration 1 to <4  $\mu$ g/ml were considered sensitive to methicillin (MSS) while methicillin resistant staphylococci (MRS) were those able to grow at concentration  $\geq$ 4  $\mu$ g/ml. Concerning MIC to vancomycin, vancomycin resistant staphylococci (VRS) were those that were able to grow at concentration  $\geq$ 16  $\mu$ g/ml. Isolates from the hospital surfaces were all sensitive to vancomycin. Staphylococci resistant to methicillin and vancomycin were more dominant in clinical isolates than the isolates from the hospital surface (Table 2). Moreover, the percentage of MRS was higher than that of VRS in clinical isolates. All tested resistant staphylococci showed

high MIC values to both methicillin and vancomycin (Figure 1). Ten percent of both SA and SE were resistant to methicillin at concentration of 32  $\mu$ g/ml. Moreover, 3 out of 50 (6%) of SA and 3 out of 40 (7.5%) of SE were resistant to vancomycin at concentration of 64  $\mu$ g/ml. Fifty percent of SA and 35% SE strains were resistant to methicillin at concentration of  $\geq$  4  $\mu$ g/ml. while 22 and 27.5% of the SA and SE strains were resistant to  $\geq$ 16  $\mu$ g/ml of vancomycin. Resistance of SS strains were relatively high as 70% of the strains were resistant to 16  $\mu$ g/ml methicillin and 100% of the strains were resistant to  $\geq$  4  $\mu$ g/ml, while 10% were resistant to 32  $\mu$ g/ml vancomycin (Figure 1).

Single-targeted PCR products that were amplified from 16S RNA, *mecA* and PVL genes were 100% congruent with those targeting the three genes collectively in multiplex PCR. Multiplex PCR was carried out for all staphylococci isolates. All isolates were positive for the 16S rRNA gene specific for staphylococci. All MRSA and MRSE were found to have *mecA* gene. However, thirty percent of the phenotypically MSSA and MSSE were found to harbor *mecA* gene. Furthermore PVL gene was detected in five percent of the isolates. PVL positive isolates were SA strains recovered from wound infection,



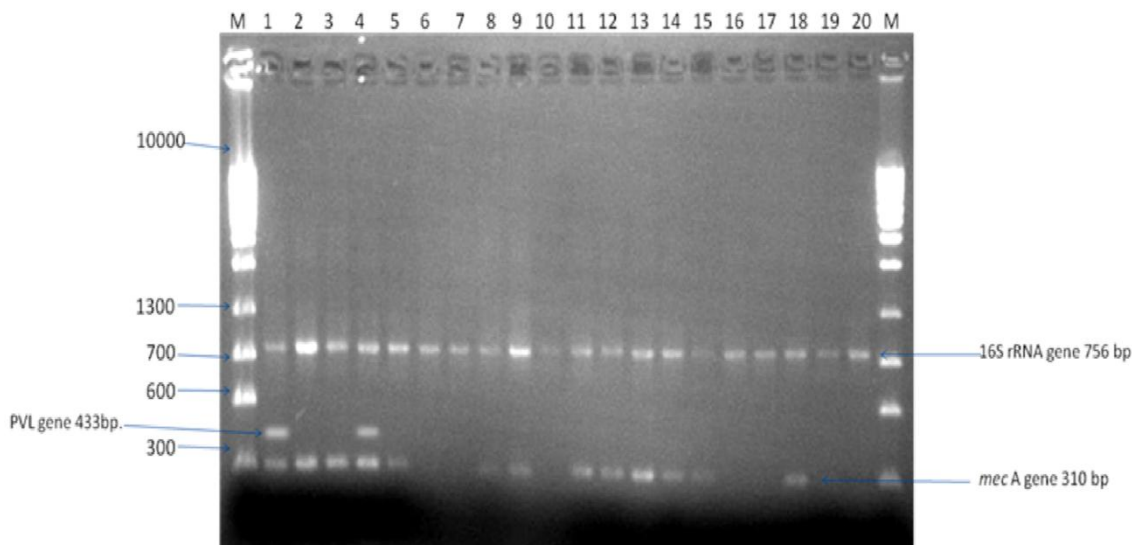
**Figure 1.** Percentage of methicillin and vancomycin resistance in staphylococci isolates recovered from their MIC data.

showing multidrug resistance and were *mecA* positive (Figure 2).

## DISCUSSION

As a result of the extensive use of antibiotics, locally isolated staphylococci showed multidrug resistance to the tested antibiotics and also had relatively high MIC to methicillin and vancomycin. Braoios et al. (2009) and McClure et al. (2006) reported a correlation between the phenotypic and genotypic results for *S. aureus* as all MRSA had *mecA* gene while the MSSA lack the gene. In the present study, the MRSA and MRSE isolates were found to have *mecA* gene thus their phenotypic characterization were similar to their genotypic characterization both confirming their resistance. However, among

the MSSA and MSSE, thirty percent of the isolates were found to contain *mecA* gene. The observation of phenotypically sensitive isolates which contain *mecA* gene highlights the possible role of horizontal gene transfer (HGT) in the dissemination of antibiotic resistance among MSSA strains. Many studies reported the transfer of *mecA* by HGT (Wielders et al., 2002; Hanssen et al., 2004). Several reports suggest that SCCmec transfer from methicillin resistant coagulase negative staphylococci (MR-CoNS) to methicillin-susceptible *S. aureus* (MSSA) occurs, although its mechanism remains unknown. MR-CoNS may thus act as a source of SCCmec for MRSA (Barbier et al, 2010). It could be concluded that phenotypic MSS might have partial SCCmec that lack the regulatory genes or it could have a mutated SCC *mec*. Further investigation is needed to determine the presence of unexpressed *mecA* gene in



**Figure 2.** A representative agarose gel electrophoresis of multiplex PCR product resulting from amplification of genomic DNA using the three primer pairs of *mecA* gene (310 bp), specific region of 16S rRNA gene (756 bp) and the virulence marker PVL (*Luk s/f-pv*) genes product (433 bp), M is 1 kbp; DNA marker. Lanes 1-5 PCR products of resistant *S. aureus*, lanes 6-10: PCR products of sensitive *S. aureus*, Lanes 11-15 PCR products of resistant *S. epidermidis* and lanes: 16-20 PCR products of sensitive *S. epidermidis*.

MSS strains. Consequently, conventional methods for the detection of MRS by disc diffusion method are not sufficient for judging resistance (Fluit et al., 2001).

Multiplex PCR is an accurate method for the detection of the resistant and virulent isolates of staphylococci and it is recommended for a rapid detection and diagnosis (McClure et al., 2006). The choice of PVL genes is based on the finding that staphylococci which contain (*luk S-F pvl*) genes showed increased disease severity of cutaneous infection (Vandenesch et al., 2003; Deurenberg et al., 2004). Furthermore, it was reported that (*luk S-F pvl*) genes were found in a high proportion (77%) in emerging community acquired staphylococcal strains (Naimi et al., 2003; Shukla et al., 2004; Naas et al., 2005).

However the present study showed that only 5% of the examined isolates harbored the PVL. The PVL<sup>+</sup> isolate was recovered from pus discharging wound. The antibiotic resistance pattern of these isolates was similar to the other isolates lacking (*luk S-F pvl*) genes, thus indicating that the presence of (*luk S-F pvl*) genes does not necessarily indicate higher potency. Similar results were reported in a study where PVL<sup>+</sup> strains were compared with PVL<sup>-</sup> mutant strains, both were found equally lethal and virulent (Said-Salim et al., 2005; Diep et al., 2006). The coexistence of PVL and *mecA* gene was demonstrated in only 5 out of the hundred examined isolates; this is in accordance with the results described by Gillet et al. (2002), Vandenesch et al. (2003) and Zhang et al. (2011) who concluded that PVL locus is carried on bacteriophage that is presently found in small proportion (less than 5%) of staphylococcal isolates

described worldwide. Accordingly, the previously described principle that PVL is always associated with virulent staphylococci and result in out breaks in soft tissue is still debatable (Zhang et al. 2011). Further studies are required to draw more conclusions regarding the potential association between *mecA*, PVL and severity of infection. A combination of conventional and multiplex PCR is essential for the detection of the potentially pathogenic *Staphylococci* including phenotypically resistant and sensitive isolates.

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