

Antimicrobial Susceptibility Patterns and Identification of Plasmid-borne Methicillin Resistant *Staphylococcus aureus*

¹Ambrina Khatoon, ²Mustafa Kamal, ¹Syed F. Hussain,
²Wasi Alam, ²Omar Rauf and ¹Syed M. Shahid

¹The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE),
University of Karachi, Karachi, Pakistan

²Department of Biotechnology, University of Karachi, Karachi, Pakistan

Abstract: A total of 115 *Staphylococcus aureus* isolates were collected from laboratories situated in different areas of Karachi and from Department of Biotechnology, University of Karachi. Each isolate screened by Gram staining, catalase test, DNase test and coagulase test. All of 115 isolates were found to be Gram positive and catalase positive. Only 96 isolates were found to be DNase positive whereas, 106 isolates hydrolyzed DNA agar. Then 106 coagulase positive *Staphylococcus aureus* selected and tested for antibiotics susceptibility by Minimum Inhibitory Concentration agar dilution method. Seven antibiotics selected which were ampicillin, gentamicin, kanamycin, ciprofloxacin, chloramphenicol, methicillin and vancomycin. Out of 106 isolates 27 were found to be multidrug resistant. Out of which 85% were resistant against ampicillin, 43% against kanamycin, 24% against gentamicin, 5% against chloramphenicol and 40% against methicillin. Only 8% were resistant to Ciprofloxacin and Vancomycin. VRSA were selected for heat induced curing. After curing vancomycin resistance was lost from all 9 isolates. Curing result of 8 MRSA showed that resistant determinant of 5 isolates were located on non-transferable DNA whereas 3 isolates exhibited plasmid-borne methicillin resistance, as the resistance was lost after curing. Plasmid isolated from 2 isolates which showed plasmid-borne methicillin resistance. Electrophoretic analysis showed that two isolates harbor a single plasmid DNA.

Key words: Methicillin Resistant *Staphylococcus aureus* (MRSA) • Methicillin Sensitive *Staphylococcus aureus* (MSSA) • Vancomycin Intermediate *Staphylococcus aureus* (VISA) • Vancomycin Sensitive *Staphylococcus aureus* (VSSA) • Minimum Inhibitory Concentration (MIC)

INTRODUCTION

Staphylococcus aureus remains one of the major bacterial pathogen whether its acquisition is health care related or in the community. It is associated with a variety of clinical infections including septicemia, pneumonia, wound sepsis, septic arthritis and post-surgical toxic shock syndrome with substantial rates of mortality and morbidity [1]. *S.aureus* has a record of developing resistance quickly and successfully to antibiotics and has overcome all the therapeutic agents that have been developed in the past 50 years. An important feature of this human pathogen is its great variability and diverse clonal type occurring at different periods and places. These clones have been particularly successful in adapting to the ever-increasing antibiotic selective pressure by renovating their genomes through both

mutation and the acquisition of exogenous genes [2]. Since the emergence of *S. aureus* strains with resistance to penicillin and methicillin in 1948 and 1961 respectively has become a well known etiological agent of a wide variety of infection [3], which was followed by the occurrence of vancomycin resistant *S.aureus* (VRSA) in June, 2002 from Michigan [4]. VRSA are addition to the burden of methicillin resistant *S.aureus* (MRSA) and are particularly difficult to treat if they are pursuing multidrug resistance. Although there is a paucity of data on the susceptibility patterns of *S.aureus* in Pakistan and prevalence of MRSA and VRSA is also unknown. However, studies on antimicrobial susceptibility have been described [5,6]. *S.aureus* intermediately resistant to vancomycin has been reported [5], but they didn't notice the presence of VRSA. For practicing physicians, clinical microbiologists and public officials, knowledge of the

local antimicrobial resistance patterns of the bacterial pathogen is essential to guide empirical and pathogen specific therapy. During this study, VRSA has been reported for the first time from Pakistan and emergence of Plasmid-borne MRSA isolates described for the first time since, 1988 [7].

Therefore, the present investigation were undertaken to obtain a picture of the *S.aureus* resistance situation of hospitals in Karachi, Pakistan with varying MRSA and VRSA frequency and thereafter to investigate the correlation between resistance with their genomes.

MATERIALS AND METHODS

Media and Culture Condition

Collection of Bacterial Samples: A total of one hundred and fifteen *S. aureus* samples were obtained from routine diagnostic laboratories, hospitals including Ehsanullah Laboratories, Mehdi Manji Laboratories, Civil Hospital, Tabba Heart Centre and from culture collection of the Department of Biotechnology, University of Karachi, Pakistan during June to August 2008. Samples were inoculated on to Luria Agar plates. Well isolated colonies were picked up and stored for further work.

Dnase Agar and Muller-Hinton Agar were used in the isolation and identification of bacterial samples. Bacterial culture was done at 37°C and curing at 43°C.

Screening and Identification of Bacteria: All bacterial samples were subjected to Gram stain, catalase, DNase and coagulase tests. All tests were performed according to standard protocols [8,9].

Antibiotic Susceptibility Testing: The susceptibility testing of 106 coagulase-positive *S.aureus* strains to seven antibiotics was carried out by Agar dilution method according to National Committee for Clinical Laboratory Standard guidelines [10]. The antibiotics included were ampicillin, kanamycin, gentamicin, ciprofloxacin, methicillin, chloramphenicol and vancomycin.

Genetic Localization of Resistance Determinants: Based on MIC determination, only those isolates were selected which showed vancomycin resistance, which were only nine and out of eight were also methicillin resistant isolates. All the nine VRSA and MRSA were selected for heat induced curing method [11], assessment followed by antibiotic susceptibility testing.

Isolation of Plasmid: Multiple drug resistant strains of *S. aureus* which showed plasmid borne methicillin resistance were selected for plasmid extraction. Plasmid DNA was extracted from mid log phase cells to produced protoplasts [12]. Protoplasts were lysed by alkaline lysis [13], followed by RNase A treatment [14].

Electrophoretic Analysis of the Plasmid DNA: Agarose gel electrophoresis of plasmid DNA of selected multi drug resistant strains of *S. aureus* was carried out on 0.8% agarose [9].

RESULTS AND DISCUSSION

All 115 *S. aureus* isolates were found to be Gram positive cocci and catalase positive where as 96 isolates were DNase positive and 106 isolates were found to be coagulase positive.

Antibiotic Susceptibility Testing: The antibiotic susceptibility of *S. aureus* isolates is described in Table 1. In this study, only 10 out of 106 isolates were found to be susceptible to all seven antibiotics tested and 27 (25.4%) isolates exhibited multidrug resistance of which 25 were found to be MRSA, 09 were VISA and 07 were VRSA. Multidrug resistance has been reported to be relatively high in African countries including Morocco, Kenya, Nigeria and Cameroon [15]. Multidrug resistance was defined as resistance to ampicillin plus three or more antimicrobial agents.

Ampicillin were the least effective antimicrobial agent with 85% resistance whereas, the ciprofloxacin and vancomycin found to be the most effective against *S. aureus* infection as mentioned in Table 1.

Susceptibility results of Ampicillin in this study are in accordance with Kalsoom and Hameed [5] another report⁵ that also found high resistance against ampicillin (92%) but contradicts with high levels of Ciprofloxacin (59%) and Gentamicin (58%). Other study Fish *et al.* [16] reported comparable findings as compared to present study. High rates of resistance with penicillins, aminoglycosides and fluoroquinolone have also been reported. In present study, only 24% isolates were resistant to gentamicin. Namias *et al.* [17] also reported *S. aureus* isolates with similar antibiotic resistance pattern to gentamicin (30%).

Oxacillin resistance for *S. aureus* isolates was 61.29% [5]. Oxacillin is primarily used for the detection of methicillin resistance. In this study 43 isolates (40%) were found to be methicillin resistance compared to

Table 1: Antibacterial resistance of *S. aureus* isolates

Antimicrobial Agents	Number of Isolates		
	Susceptible	Intermediates	Resistant
Ampicillin	14	2	90
Kenamycin	44	16	46
Methicillin	63	–	43
Vancomycin	49	48	9
Ciprofloxacin	97	–	9
Gentamicin	80	–	26
Chloramphenicol	69	–	37

Table 2: Resistance pattern for each class of antibiotic against MRSA

Structural class of Antibiotic	Representative Antibiotics %	Resistance%	Susceptibility
β -lactam	Amp, met	98	2
Aminoglycosides	Gen, Kan	60	44
Fluoroquinolone	Cip	21	78
Glycopeptides	Van	19	40
Phenylpropanoid	Chl	48	52

Amp, ampicillin; Cip, ciprofloxacin; Chl, chloramphenicol; Gen, gentamicin; Kan, kanamycin; Met, methicillin; Van, Vancomycin.

Antibiotic susceptibility of *S. aureus* against

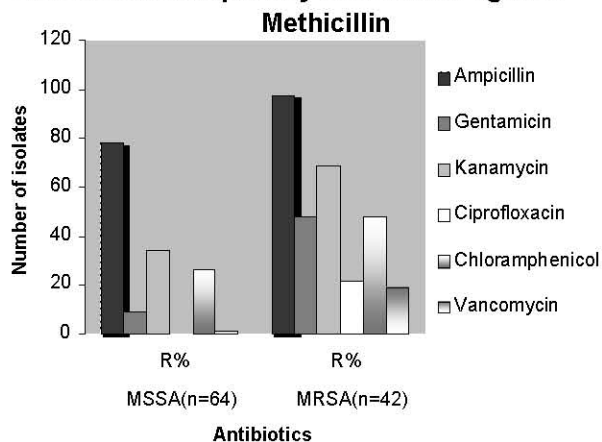


Fig 1: Antibiotic susceptibility of *S. aureus* (MSSA and MRSA)

R= Resistant

MSSA = Methicillin Sensitive *S. aureus*

MRSA = Methicillin Resistant *S. aureus*.

Mahmood *et al.* [6] who found that methicillin resistance among 48 out of 246 isolates (19.51%). According to above data it can be concluded that MRSA isolates increased from 19.51% in 2001 to 40% in 2008 in Pakistan. The prevalence of MRSA has increased worldwide as is evident from many surveillance studies [18-22]. However, there are considerable differences between individual countries, the very highest rate of methicillin resistance (64%) among *S. aureus* isolates have been noted in Western Pacific regions, including Korea [23]. SARISA study group [24] found the expected wide geographical variation of MRSA with low levels in hospitals in

Northern European, increasing levels in middle European countries, United States, New Zealand and Australia and very high levels in Southern European countries as well as in parts of United States, Asia and South Africa.

There was a relationship between methicillin resistance to other antibiotics as noted in previous investigations [18, 23]. In present study, 13 out of 43 MRSA (30%) were found to be multidrug resistance as shown in Table 2. The proportion of MRSA resistant to β -lactam (98%) and aminoglycosides (60%) were found to be very high as mentioned in Table 2, 48% MRSA were resistant to phenylpropanoid (chloramphenicol) and 19% were resistant to glycopeptide (vancomycin). In general, elevated rates of multidrug resistance may emerge from diverse isolates of *S. aureus* under antimicrobial pressures or as a result of widespread person-to-person transmission of multidrug resistant isolates [25].

About 78% MRSA isolates were susceptible to fluoroquinolone. The most effective antibiotic found against MRSA was ciprofloxacin analogous with the data from multicenter drug study (South Africa). But ciprofloxacin found to be least effective against MRSA with proportion of resistance increased from 53% in 1994 to 92% in 2004 in Kuwait hospital [26]. An increase in the proportion of ciprofloxacin resistance (from 4% in 1998 to 75.9% in 1999) was also observed in MRSA isolated in Australian teaching hospital [27]. Thus, MRSA isolates showed much higher rates of resistance to the others antimicrobial agents than did MSSA isolates and were resistant to multiple agents analogous to the work of SARISA study group [24], as briefly described in Figure 1.

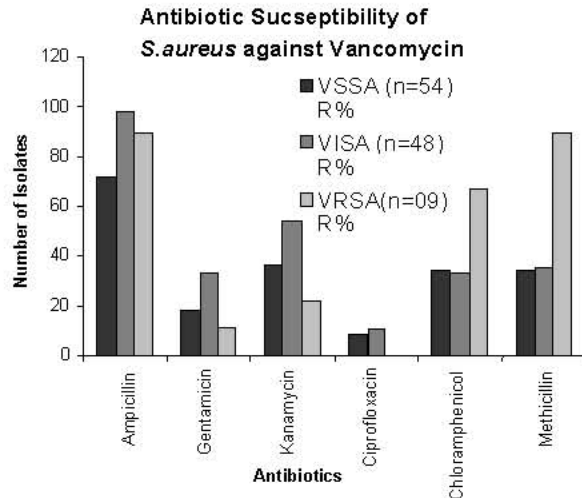


Fig. 2: Antibiotic susceptibility of *S. aureus* isolates (VSSA, VISA and VRSA)

VSSA = Vancomycin Sensitive *S. aureus*

VISA = Vancomycin Intermediate *S. aureus*

VRSA = Vancomycin Resistant *S. aureus*.

Table 3: Genetic localization of vancomycin and methicillin resistance determinants

Strain No.	Growth After Curing Vancomycin	Methicillin
AKM01	-	+
AKM23	-	+
AKM67	-	+
AKM68	-	+
AKM71	-	+
AKM72	-	+
AKM76	-	-
AKM77	-	-
AKM79	-	-

During this study, vancomycin resistance has been reported for the first time from this region and 8% of *S. aureus* were found to be VRSA. Kalsoom and Hameed [5] didn't find any VRSA during their studies but 38% isolates were found to be vancomycin intermediate as we have also found 45% VISA. Ciprofloxacin was also found to be most effective antimicrobial agent against VRSA and exhibited 100% susceptibility as mentioned in Figure 2. Whereas, only 8% VSSA and 10.4% VISA were resistant to ciprofloxacin. VRSA isolates showed much lower rates of resistance against ampicillin, kanamycin and gentamicin than did MRSA isolates. Accordingly, 88% VRSA and 35% VSSA were resistant to methicillin.

Genetic Localization of Resistance Determinants:

The genetic instability of vancomycin in nine VRSA isolate has been confirmed by curing. These isolates carried plasmid mediated resistance to vancomycin analogous to international work as shown in Table 3.

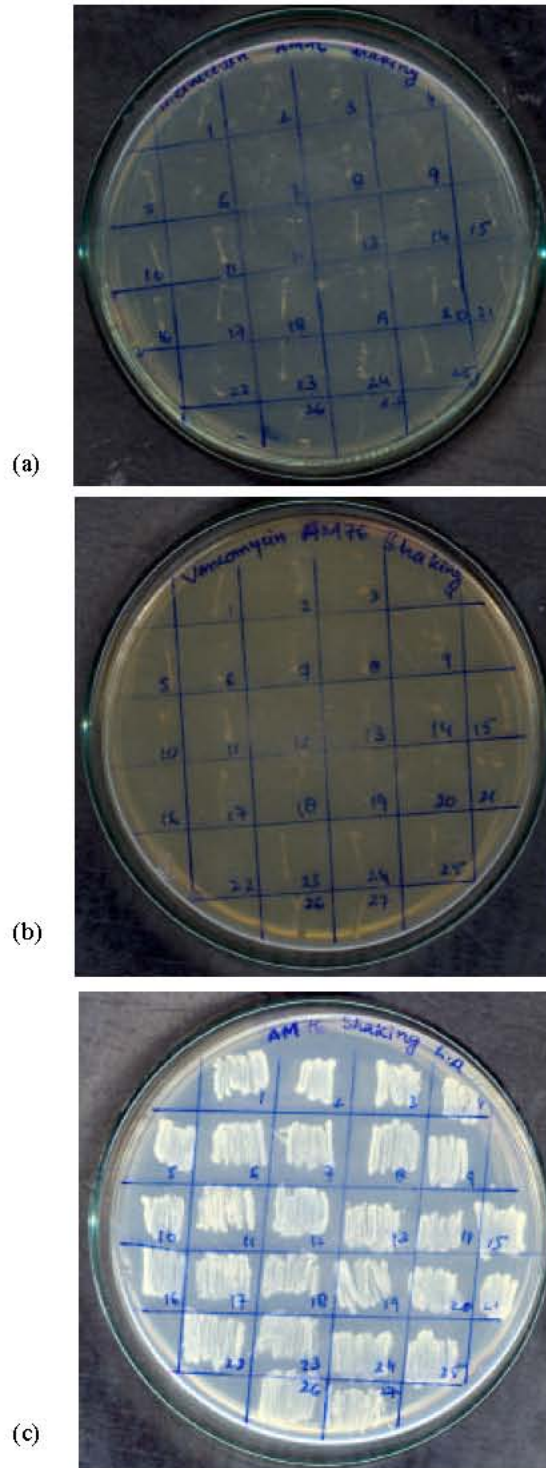


Fig. 3: Antibiotic susceptibility of AKM76 after curing

(a): MHA-plate containing Methicillin.

(b): MHA-plate containing Vancomycin no growth occur after 24hrs in a and b.

(c): Luria Agar plate having no antibiotic; strains of AKM76 grow normally.

Whereas, curing result of 8 MRSA indicated that resistant determinant in 6 out of 9 MRSA were located in non-transferable DNA and 3 isolates exhibited plasmid-borne methicillin resistance. Loss of methicillin resistance from 3 isolates (AKM76, AKM77 and AKM79) shown in Table 3, give striking result because there are more supporting evidence that *mecA* genes are located on chromosome [28, 29]. Antibiotic susceptibility of AKM76 also shown in Figure 3(a-c). *mecA* genes are responsible of methicillin resistant in *S. aureus*. There are many possibilities of these results. One possibility for this finding could be the β -lactamase plasmid may provide a temporary insertion site for the *mec* containing transposons. There is one report somewhat controversial because it has never been confirmed of a *mecA* containing transposons, Tn4241, residing in an insertion site on the β -lactamase plasmid p1524 [7].

Genetic instability could be possible if resistance genes are recently integrated into the chromosome as a result of transposition or plasmid insertion but at a low frequency [30,31] MRSA was born when it acquired a large unique class of mobile genetic element known as the SCCmec [32, 33]. For movement, SCCmec carries two specific genes designated cassette chromosome recombinases of the invertase family [32]. In the presence of these recombinases SCCmec integrates into the chromosome, in the correct orientation and is also precisely excised from the chromosome [33].

It also possible that these isolate which show loss of methicillin resistance may lack *mecA* gene but contain normal penicillin binding protein with modified penicillin binding capacity [33]. Electrophoretic analysis shown that the selected strains found to harbor a single plasmid DNA (Figure 4).

In summary, *S. aureus* has always been able to conquer the antibiotics developed it is also clear that it will acquire resistance to any novel antibiotics developed in the future. Therefore we cannot rely solely on the development of new chemotherapeutic agents. The best way to solve this problem is to control the consumption of antibiotics in hospitals. It is necessary for government and health care authorities to take an active role. They must implement educational programs, encourage better infection control and prevent antimicrobial usage and fund research.

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