ORIGINAL ARTICLE

Current Pattern of Antibiotic Resistance in *Staphylococcus Aureus* Clinical Isolates and the Emergence of Vancomycin Resistance

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ABSTRACT

Objective: To determine the pattern of antibiotic resistance in the clinical isolates of *Staphylococcus (S.) aureus*, Methicillin Resistant *S. aureus* (MRSA) and define the possible emergence of Vancomycin resistant *S. aureus* (VRSA) in Karachi.

Study Design: An observational study.

Place and Duration of Study: Essa Laboratories and Department of Molecular Genetics, Ziauddin Hospital, from January to December 2009.

Methodology: Staphylococcal isolates from different clinical specimens, pus, urine, blood, high vaginal swab and other secretions received at Ziauddun laboratories and Dr.Essa laboratories were collected. The specimens were inoculated on blood agar, MacConkey agar and Chrom agar. Antibiotic susceptibility to conventional antibiotics was done by disc diffusion, and E-test. Methicillin resistance was tested by using Oxacillin and Methicillin disks and confirmed by gold standard PCR for presence of mecA gene. All MRSA strains were subjected in addition to Vancomycin screen agar test.

Results: Out of the 450 *S. aureus* isolates 174 (38.6%) were found to be MRSA. In those isolates, high resistance was found to Cefixime (100%) Doxicycline (100%) Oxacillin (96.5%) Gentamicin, (96.3%), Timethoprim/Sulfametoxazole (95.6%) Chloramphenicol (93%) Tobramicin (81.03%), Ofloxacin (72.4%) and Ciprofloxacin (63.7%). Low resistance was found to Ceftazidine (36%), Amoxicillin/Clavulanate (32.7%), Fosfomycin (31%), Cefroxime (24%), Amikacin (17.2%) and Meropenem (13%). One isolate was found to be Vancomycin resistant (MIC 32 µg/ml). Four isolates had intermediate resistance, with two strains having MIC of 16µg/ml and two having MIC of 8µg/ml. These strains were also resistant to all the other tested antibiotics except Linezolid to which all isolates were susceptible.

Conclusion: Antibiotic resistance to all the conventionally used antibiotics was high in the tested isolates. All the strains were susceptible to Linezolid which is an expensive alternative with adverse side effects. Judicious use of antibiotics focused on the compliance and formation of antibiotic policy guide lines is highly recommended.

Key words: *Methicillin resistant Staphylococcus aureus (MRSA). Vancomycin. Multidrug resistance. Linezolid.*

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a well known pathogen of infections acquired in hospitals, and is now acknowledged as a community pathogen as well.[1] After Penicillin was introduced in 1941, *S. aureus* became resistant to penicillin in a few years by producing an enzyme beta-lactamase that caused degradation of the antibiotic making it ineffective.[2] MRSA organisms are in general resistant to multiple antibiotics including aminoglycosides, macrolides, fluoroquinolones, Clindamycin, Trimethoprim/Sulfamethaxozole, Chloramphenicol and beta-lactamases due to the acquirement of the mecA gene. This may occur as a spontaneous genetic mutation or involve acquirement of a genetic material such as plasmid, transposon, integron, or a gene cassette.[3] The resistance may be due to the attainment of gene by horizontal transmission by conjugation of genetic material from *Staphylococcuc sciuir*.[4] Geographic clustering of closely related genotypes has been described in several `studies signifying that the spread is frequent and further than the limitations of hospitals.[5]

In the early 1970's, MRSA resistant to orally administered antibiotics and sensitive only to the antibiotic Vancomycin emerged. Off late, there has been a mounting resistance to Vancomycin by species such as Enterococcus.[4] The appearance of Vancomycin resistance in Enterococcus gave rise to the likelihood of horizontal transmission by conjugation of resistance genes to *S. aureus*. In laboratory procedures, Vancomycin resistance has been transferred from *Enterococcus faecalis* to *S. aureus*, which steadily expresses the phenotype.[4]

In May 1997 the Center for Disease Control and Prevention (CDC) reported the first incidence of ineffectiveness of Vancomycin in Japan. Strains of Vancomycin intermediate *Staphylococcus aureus* (VISA) with Vancomycin MIC of 8µg/ml have been reported from Japan, France and Germany.[6-8] They are invariably also resistant to other glycopeptides used in clinical practice (Teicoplanin) and therefore are depicted as GISA (glycopeptide intermediate *Staphylococcus aureus*).[6] Hence a constant close watch of resistant patterns should be considered in

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order to direct antibiotic therapy which may be needed to be tailored. In the clinical laboratory, the standard means of identifying Methicillin resistance is according to NCCLS.[9] Recently, molecular typing methods have been optimized to detect the mecA gene in MRSA. PCR technique shows a high level of coordination with susceptibility tests and allows a precise categorization of highly resistant and borderline-resistant strains.[10] The main objective of this study was to screen the current antibiotic resistant pattern of clinical isolates of *Staphylococcus*, the frequency of MRSA and the possible presence of Vancomycin resistance in Karachi.

METHODOLOGY

The study was carried out at The Essa Laboratory and the Department of Molecular Genetics, Ziauddin Hospital, Karachi. A total of 450 staphylococcal isolates were investigated for a period of one year from January to December 2009. These strains were collected from various clinical specimens including pus, urine, wound swabs, blood, secretions, high vaginal swab, and CSF obtained from major laboratories in Karachi. Specimens are processed within 2 hours of collection by standard microbiology techniques. Sheep blood agar and mannitol salt agar were used for all samples except urine which was only inoculated on CLED agar (Oxoid). The agar plates were incubated at 35C for 18-24 hours in aerobic atmosphere (NCCLS, 2003).

Plates were examined for *Staphylococcus* by colony morphology, and Gram staining, yellow to cream or occasionally white 1-2 mm in diameter, slightly raised colonies after overnight incubation were seen which on gram staining showed purple grape like clusters. Some strains show beta-hemolysis on blood agar plate. Chrom agar Pink to mauve colonies of coagulase positive staphylococci were seen in 24 hours (CHROMagar Rambach, France[11]).

Catalase Test was performed by adding 3% hydrogen peroxide to a colony on an agar plate. A slide coagulase test of all 450 isolates was performed by emulsifying few pure colonies of staphylococci from blood agar on undiluted rabbit plasma. Tube coagulase test was performed diluting the plasma in freshly prepared normal saline (1:6). Three to four pure colonies were emulsified in 1 ml of diluted plasma and the tubes were incubated at 37C. Readings were taken at 1h, 2h, 3h, and 4 h and further incubated overnight at room temperature if no clot formation was observed. *S. aureus* ATCC 29123 was used as the control strain.

The colonies were picked with sterile cotton swabs and dipped into 0.5 McFarland turbidity standard to make direct colony suspension of the isolates. The suspension was streaked on to Mueller-Hinton agar (MHA) plates. These plates were then incubated for 24 hours. *S. aureus* ATCC 29123 and *Enterocococcus faecalis* ATCC29212 strains were used as Vancomycin susceptible controls and *E. faecalis* ATCC 51299 as Vancomycin resistant control.

The zone interpretation of each antimicrobial agent was done in accordance with NCCLS (2003) guidelines. The technique used for antimicrobial susceptibility testing for methicillin resistance was different from other anti-microbials. The microbial suspension after comparing with 0.5 McFarland turbidity standards was streaked on to Mueller-Hinton agar supplemented with 4.5% sodium chloride as recommended by Clinical and Laboratory Standards Institute (CLSI, 2005).[12] The plates were allowed to dry for 3-5 minutes before the disks were used. The Methicillin disc (5 µg) was used for susceptibility testing. The results were interpreted according to NCCLS guidelines; a zone of < 10 mm was considered resistant, and of > 13 mm was considered susceptible.

The 174 isolated MRSA strains were checked for sensitivity to Vancomycin using E-strip test (AB Biodisk USA). Bacterial lawns were made on MHA and the E-strips were positioned on the agar surface with sterile forceps, and incubated at 37C for 24 hours. The MIC for Vancomycin from E test strip was noted according to the manufacturers' guidelines (AB –biodisk, USA) and the NCCLS standards of 2003.

All PCRs were carried out adhering to the standard precautions to avoid contamination. These included preparation of reaction mixtures in a Biosafety hood level 2, use of face masks, gloves and laboratory coats.

The reagents were reconstituted, and stored according to the manufacturer's instructions given in the literature. Primers were purchased and dissolved in sterile ultrapure water to the required concentration before storing at-20C

Bacterial DNA was extracted using QIAgen DNA min (Spin Column) Kit according to the manufacturer's instruction. DNA was extracted from 5 ml of an 18-h broth culture. Cells were collected from the cultures by centrifugation at 1,000 x g for 10 minutes, resuspended in phosphate-buffered saline with 100 µg of lysostaphin (Sigma) per ml, and incubated at 37C for 0.5h or until viscous. DNA from all preparations was subsequently extracted with QIAgen DNA protocol. Isolated DNA samples were dissolved in TE buffer [10 mM Tris chloride-1mM EDTA (pH 8.0].[13]

The PCR was performed according to the procedure of Geha *et al.*[10] The presence of mec A (gene coding for penicillin binding protein 2A) was detected by PCR using forward primer (5' GAT GAA ATG ACT GAA CGT CCG ATAA3' and reverse primer 5'CCA ATT CCA CAT TGT TTC GGT CTA A3).

In all strains these primers give rise to a PCR product of 310 base pairs. The PCR reaction mixture was prepared by using 2.5 µl 10x buffer, 0.2 mM dNTPs, 4 U Taq DNA

polymerase, 2.5 mM $MgCl_2$, 50ng DNA template, 1.5 µl of mec A1 primer and 1.5 µl of mec A2 primer. The volume was made up to 25 µl with 2.5 µl of ultra pure water. DNA amplification was carried out in a Perkin Elmer thermocycler with the following thermal cycle profile: initial denaturing at 94 C for 4 minutes was followed by 35 cycles of amplification (denaturation at 94 C for 45 s annealing at 58 C for 45 s and extension at 72 C for 60 s) ending with a final extension at 72 C for 2 minutes.

S. aureus ATCC 29213 refrence strain was used as the mecA negative control strain and *S. aureus* ATCC43300 as mecA positive control strain.

The PCR products, alliquot (10 µl) were loaded on an ethium bromide gel (0.5 µg/ml; Sigma Ltd. USA) 3% agrose (Sigma) in TrisBorate EDTA (TBE) buffer, with known markers and run at 90V for one hour before visualization under Ultra Violet Biodoct digital imaging system (UVP, Inc. Cambridge UK) for the presence of 310 base pair PCR products.

The study was an observational one and data were summarized as count and percentages. SPSS version 12 was used for data analysis.

RESULTS

The *S. aureus* strains were confirmed coagulase positive by the tube coagulase test. Of those, 174 (Table I) isolates were identified as MRSA as determined by susceptibility to Methicillin discs and growth on Methicillin Chrom agar. Chromogenic MRSA medium permits the direct detection and identification of MRSA through incorporation of specific chromogenic substrates and Cefoxitin and showed mauve colored colonies. The prevalence of MRSA by Methicillin disc diffusion was (38.6%) and Oxacillin disc was (37%) in our study. Most of the isolates were obtained from pus and wound swabs while next in frequency were urine, blood and high vaginal swabs.

Table I: Frequency distribution of *S.aureus* and MRSA.

	MRSA f (%)	*S.aureus* f (%)
Pus and wounds swab	93 (57.4)	162 (36)
Urine	42 (33.3)	126 (28)
High vaginal swab	31 (46.96)	66 (14.7)
Ear swab	0 (0.00)	15 (3.33)
Blood	08 (22.22)	36 (8)
Urethral swab	0 (0.00)	19 (4.22)
Eye swab	0 (0.00)	26 (5.78)
Total	174	450

Later isolated methicillin resistant strains of *S. aureus* (n=174) were tested specifically against different concentrations of vancomycin by the E-strip test. One strain was found to be Vancomycin Resistant *S. aureus* (VRSA) and showed MIC of 32 µg/ml. Four strains were found to be Vancomycin intermediate (two strains with MIC of 16 µg/ml and two strains with MIC of 8 µg/ml. Disc diffusion test showed that all Vancomycin resistant staphylococcal isolates have shown antimicrobial resistance to most of the community used antimicrobials. Multiple drug resistance was more common in MRSA group as compared to MSSA (Table II).

Table II: Antibiotic resistance pattern of MRSA and MSSA (total 450).

Antibiotics	MRSA	MSSA
Penicillin	174	272
Cefixime	174	276
Doxicyclin	174	243
Azetreonam	174	276
Nalidixic acid	174	276
Genatamicin	168	135
Septran	167	260
Chloamphenicol	162	243
Tobramycin	141	16
Ofloxacin	126	166
Ciprofloxacin	111	139
Amoxicillin	99	105
Cefrotaxime	84	23
Ceftriaxone	78	23
Ceftoxitin	63	19
Velosef	64	55
Fosfomycin	54	16
Cefroxime	42	20
Amikacin	30	17
Meropenem	24	6
Vancomycin	1	0
Linezolid	0	0

The results of PCR show excellent agreement with no exceptions. PCR showed the presence of mecA gene in all the 174 tested isolates. The mecA specific PCR product of 310 base pairs was seen in all MRSA strains (Figure 1).

The PCR amplification of the mecA fragment was run on a ethium bromide stained agarose gel and on visualization under UV light an amplicon of 310 base pairs was seen on all of the tested samples.

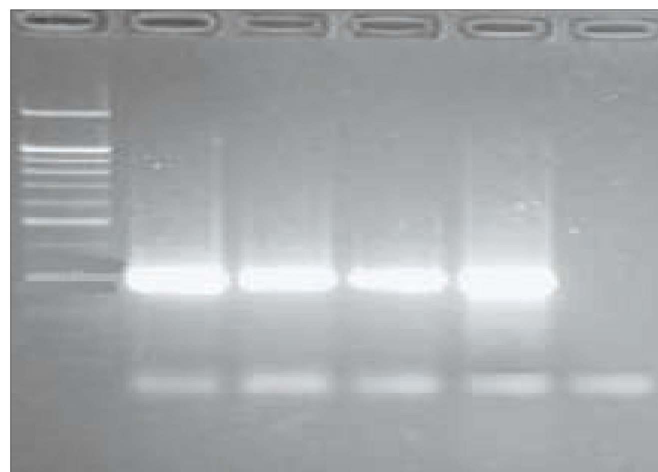

Figure 1: Agrose gel of mecA PCR products.
(Lane 1: DNA markers; Lane 2-5: mecA positive; Lane 6: mecA negative controlle).

DISCUSSION

MRSA is a pathogen which has the dangerous combinations of being virulent, resistance to conventional treatments and an ability to disseminate at large.[14,15] As *S. aureus*

strains continue to evolve there is an irrefutable need to fully characterize them. In the standard laboratory, prompt identification of the staphylococcal strains up to species level is done by catalase, coagulase, and other biochemical tests. It is possible during routine screening by slide coagulase test, many strains of *S. aureus* are missed due to their sub-optimal sensitivity. Therefore, the main criterion used for the identification of *S. aureus* in this study was tube coagulase test.

The last decade has seen a startling rise in cases of MRSA in Pakistan.[16] The incidence of MRSA differs considerably from one region to another and among hospitals in the same city but the data on the prevalence of MRSA strains in Pakistan is limited. In some hospitals MRSA accounts for < 10% of all *S. aureus* isolates in others it is reported up to 65%.[17] In 1999, MRSA accounted for > 50% of *S. aureus* from patients in ICU in the National Nosocomial Infection Surveillance system. By 2004, the figure increased to 63%.[18] Different reports have shown in the last seven years, resistance in MRSA has increased at a steady pace to most antimicrobial agents such as Gentamicin from (69%) in 1996 to 88% in 2003; Ciprofloxacin from 87% in 1996 to 94% in 2003 and for Rifampicin from 20% in 1996 to 60% in 2003. Another study carried out at the Aga Khan University Hospital Karachi reported similar resistance pattern as in this study.[19] According to this study high resistance was observed against Cotrimoxazole (59%), Tetracycline (82%) and Clindamycin (79%). Methicillin resistance correspondence with the presence of mecA, the gene that codes for a penicillin binding protein (PB2a) not affected by these drugs. All the 174 isolates showed the presence of mecA gene by PCR. The results of PCR showed excellent agreement with susceptibility tests. It has been shown that Staphylococci that grow on Mueller Hinton agar containing 4% NaCl and 6 µg/ml Oxacillin typically are mecA positive.[20] One of the MRSA strains showed Vancomycin resistance (VRSA), (MIC 32 µg/ml) isolated from the pus of a 63 year old male. This isolate was found to be resistant to several other antimicrobials such as Gentamycin, Tobramycin, Amikacin, Norfloxacin, Ciprofloxacin, Erythromycin and Tetracycline.

The emergence of glycopeptides resistance is of great concern, Hakim *et al.* reported the presence of 13% VISA strains, resistant to 30 µg/ml of Vancomycin.[21] There are reports of emergence of Vancomycin resistant *Staphylococcus aureus* from India.[22] This is the first time Vancomycin resistance has been reported from Pakistan. The development of antibiotic resistance in developing countries is due to the unjustified use of antibiotics, over the counter availability without prescription and uncontrolled use in agriculture, animal husbandry and fisheries. This emergence of VRSA/ VISA may be due to building of selective pressure of Vancomycin. Vancomycin, a glycopeptide is currently the main antmicrobial agent available to treat life-threatening infections with MRSA.[23] The development and subsequent emergence of resistance to vancomycin is perceived as a demanding situation adding on to the already difficult to treat MRSA.

The mechanism of Vancomycin resistance has been extensively studied with the first clinical VRSA strain Mu50.[24] Biochemical and transmission electron microscopy (TEM) examination of the Mu cell suggested that it produced increased amount of peptidoglycan. It is needed to control the spread of these resistant strains through infection control programmes,[25] and continuous monitoring of the drug resistance pattern and restrict the use of Vancomycin unless it is imperative.

There were some limitations to the result of this study. A larger sample size would have had a greater probability for increased number of observations. This was restricted due to limited resources and time. Nevertheless the study effectively highlights the increased frequency and changing pattern of antibiotic resistance in Karachi.

CONCLUSION

This study has reported the emergence of VRSA and VISA for the first time in Karachi. There should be a continuously monitoring of the resistance pattern of the multidrug resistant *S. aureus*. Combination therapy should be against MRSA. For surveillance programmes all strains with Vancomycin MIC 4 µg/ml should be further characterized, this will help the Health authorities for prompt action against future development of the resistant stains of *Staphylococcus*.

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