Glycopeptide Sensitivity Patterns in Staphylococci Isolated from Clinical Specimens in a Tertiary Care Hospital

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Staphylococci are among the most important and common human pathogens worldwide. Their resistance to antibiotics is increasing. The glycopeptide antibiotics (vancomycin and teicoplanin) are the last resort to treat serious infections caused by these bacteria. During the last decade the strains of staphylococci have developed intermediate levels of resistance to teicoplanin and vancomycin. The objective of this study was to establish the prevalence glycopeptide resistance among clinical isolates of staphylococci in Shaikh Zayed Hospital, Lahore, and comparison of antimicrobial sensitivities of vancomycin and teicoplanin among these isolates. 75 (Seventy five) consecutive staphylococci isolated from clinical specimens received in the laboratory were collected and their sensitivity to antibiotics was tested by National Committee for Clinical Laboratory Standards (NCCLS) disk diffusion method. Results show that all staphylococci were sensitive to the glycopeptide antibiotics. This indicates that no high-level resistance to glycopeptide antibiotics in these organisms is present in our hospital. However, the emerging resistance in staphylococci against these drugs worldwide necessitates strict surveillance of these organisms, institution of effective infection control policies and judicious use of antibiotics.

Key words: Glycopeptides. Vancomycin. Teicoplanin. Glcopeptide resistance. Staphylococci. Vancomycin-intermediate *Staphylococcus aureus*(VISA). Glycopeptide-intermediate *Staphylococcus aureus* (GISA).

Infectious diseases have been a scourge since the dawn of civilization. Global spread of infectious diseases began in the 16th century[1]. To make matters worse, bacterial pathogens have become increasingly resistant to a variety of antibiotics[2]. Of great concern is the increasing incidence of infections caused by gram-positive bacteria with acquired multidrug resistance as they are extremely difficult to treat[3]. Antimicrobial resistance in gram-positive cocci has achieved its greatest prominence in the previous 15 years or so. One of the more important problems of antimicrobial resistance in gram-positive cocci include methicillin resistance and multidrug resistance in staphylococci[4].

Staphylococcus aureus has been recognized as a major human pathogen ever since Sir Alexander Ogston first proposed, in the 1880s, that it was the major cause of wound suppuration[5]. The incidence of infections caused by coagulase-negative staphylococci (CoNS) has increased dramatically in recent years[6].

With the global spread of methicillin-resistant strains, glycopeptide antibiotics have become the mainstay of chemotherapy in both coagulase-negative staphylococci as well as *Staphylococcus aureus* infections worldwide[7,8]. Susceptibility of staphylococci to vancomycin and other glycopeptides appears to be waning[9]. The decrease in vancomycin susceptibility is accompanied, in most cases, by decreased susceptibility to another glycopeptide, teicoplanin, prompting to use the terms glycopeptide-intermediate *Staphylococcus aureus* (GISA) and glycopeptide-intermediate *Staphylococcus species* (GISS) rather than vancomycin-intermediate *Staphylococcus aureus* (VISA)[10].

Heterogeneous vancomycin-resistant *Staphylococcus aureus* (hetero-VRSA) is a preliminary stage that allows development into vancomycin-resistant *Staphylococcus aureus* (VRSA) upon exposure to vancomycin. They can't be detected by disk diffusion antimicrobial sensitivity testing in the laboratory and are also cause of therapeutic failure of vancomycin[11,12]. However, high-level resistance can be detected by disk diffusion method of antimicrobial sensitivity testing[13].

In staphylococci, the exact mechanism of resistance that results in elevated MICs of vancomycin and teicoplanin is unknown[14,15] although it likely involves alterations in the cell wall and hyperexpression of penicillin-binding proteins (PBPs)[16]. The exact mechanism of vancomycin resistance in hetero-VRSA has also not been determined and very little epidemiological data is available about them[17].

Vancomycin was introduced in Pakistan more than a decade ago, however, it has come into significant clinical use only in the last five years (personal communications, Eli Lilly Pakistan Limited). Teicoplanin was launched in 2000 (personal communications, Aventis Pharma Pakistan Limited). In the scenario of emerging resistance in staphylococci against these drugs worldwide, it was important to evaluate sensitivity of vancomycin and teicoplanin against these organisms in Pakistan. This study looked into the sensitivity of the clinically most important genus i.e. *Staphylococcus* to vancomycin and teicoplanin in Shaikh Zayed Hospital Lahore.

Materials and methods:

Seventy five consecutive staphylococci isolated from clinical specimens received in the laboratory were collected. All the specimens received in the laboratory were processed according to the standard laboratory procedures being carried out in the laboratory. The clinical

isolates were identified as *Staphylococcus aureus*, and coagulase-negative *Staphylococcus species*. Only the bacterial isolates from specimens sent for culture and sensitivity from the clinical departments were included in the study. Bacterial isolates expected to be pathogenic were included while bacterial isolates believed to be commensals e.g. coagulase-negative staphylococci from skin and wound swabs were excluded from the study. Isolates belonging to different species (i.e. *Staphylococcus aureus* or coagulase-negative staphylococci) obtained from the same patient were included. An isolate of the same species (i.e. *Staphylococcus aureus* or coagulase-negative *Staphylococcus species*) as previously obtained from the same patient was excluded from the study.

Specimens received in the laboratory were inoculated on blood agar, chocolate agar, and MacConkey agar. Blood cultures were first inoculated in tryptic soy broth (TSB) for 48 hours and then on solid media as mentioned above. All the plates were incubated at 37°C and read after 18 to 24 hours. Plates showing no growth were reincubated and read after 24 hours. No growth was declared after 48 hours of incubation. Urine cultures were inoculated on cystine lactose electrolyte-deficient agar (CLED agar), incubated at 37°C and read after 16 to 24 hours. 'No growth' was declared after 24 hours of incubation. Colony counts of $\geq 10^5$ colony-forming units (CFUs) per ml of urine were taken significant.

Laboratory's standard operating procedures for the identification of these bacteria included colonial morphology, Gram stain, catalase test, deoxyribonuclease (DNase) test, and bile-esculin test. Gram-positive cocci were selected on the basis of colonial morphology. These were confirmed by gram stain and then subjected to catalase test (Hydrogen peroxide, Merck). Catalase-positive gram-positive cocci were labeled as staphylococci and subjected to DNase test (DNase agar, Oxoid). DNase-positive strains were labeled as *Staphylococcus aureus*, and negative as coagulase-negative staphylococci[18-20].

Staphylococcal clinical isolates collected in the laboratory were stored in TSB (Oxoid) containing 15% (v/v) glycerol[13,18] 5 to 10 colonies of each bacterial isolate were picked with sterilized wire loop and inoculated into the 15% TSB glycerol broth. Stock cultures were frozen at -70°C in a freezer located in an area of the laboratory to which there was limited access[21].

Loopful of the stored 15% TSB glycerol broth cultured the collected strains stored at -70°C. The isolates were subcultured onto blood agar and MacConkey agar plates. Plates were incubated at 37°C overnight to recover the bacteria. The (frozen cultures) strains were subcultured twice prior to testing[13]. All the recovered strains were characterized again by standard clinical laboratory methods as mentioned previously.

The sensitivity testing was carried out by disk diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines. Direct colony suspension method of preparing a standardized inoculum of 0.5 McFarland turbidity standard was followed. Antimicrobial sensitivity testing was preformed by disk diffusion method and results were read after 24 hours incubation at 35-37°C. For oxacillin susceptibility testing the staphylococci were inoculated on Mueller-Hinton agar plates. After placing oxacillin 1µg disk, the plates were incubated at 35°C and the results were read after 24 hours[13].

For staphylococci the breakpoints of sensitivity of vancomycin and teicoplanin were ≥15mm and ≥14mm respectively. NCCLS recommends that any staphylococcal strain with a zone diameter of 14mm or less should be tested by an MIC method. No such strain was obtained in this study. Therefore, MIC determination was not carried out. *Staphylococcus aureus* American Type Culture Collection (ATCC) 25923 was included as sensitive test organism. Quality control testing was performed daily. Zone diameters were considered acceptable according to NCCLS recommendations[13].

Results:

In total, 75 consecutive gram-positive cocci belonging to the *Staphylococcus* genus were collected from clinical specimens received in the laboratory in a tertiary care, university teaching hospital (Shaikh Zayed Hospital Lahore). The organisms were identified according to the standard operating procedures of the laboratory. Antimicrobial sensitivity testing was done according to the recommendations of National Committee for Clinical Laboratory Standards[13]. The organisms were classified into five groups as follows:

1. Methicillin-resistant *Staphylococcus aureus* (MRSA)
2. Methicillin-sensitive *Staphylococcus aureus* (MSSA)
3. Methicillin-resistant *Staphylococcus species* (MRSS)
4. Methicillin-sensitive *Staphylococcus species* (MSSS)

Of these 75 isolates, MRSA were 40%, MSSA 40%, MRSS 6.7% and MSSS 13.3% (Table I).

Table I: Isolates (n=75)

Isolate	n=	%age
MRSA	30	40.0
MSSA	30	40.0
MRSS	5	6.7
MSSS	10	13.3

MRSA, MSSA, MRSS, MSSS: See Text

The majority, n = 43 (57.3%) of the organisms were isolated from blood while n = 16 (21.3%) were isolated from pus, n = 9 (12%) from urine, n = 4 (5.3%) from sputum and n= 3 (4%) were isolated from fluids (Table II). Distribution of organisms of each group isolated from different sources is also given in Table II. 93.3% of CoNS and 48.3% of *Staphylococcus aureus* were isolated from blood.

Table II: Isolates and Source (n=75)

Isolate	Blood	Fluid	Pus	Sputum	Urine	Total
MRSA	18	1	5	3	3	30
MSSA	11	2	10	1	6	30
MRSS	4	-	1	-	-	5
MSSS	10	-	-	-	-	10
Total	43	3	16	4	9	75

Among the 43 bacteraemic strains isolated in this study, *S. aureus*, n=29(67.4%) comprised the largest group; 62% of these were methicillin-resistant. CoNS, n=14 (32.6%) were second in frequency and of these 28.6% were methicillin-resistant (Table III).

Table III: Bacteremia Isolates (n=43)

Isolate	n=	%age
S.aureus	29	67.%
MRSA	18	62.1
MSSA	11	37.9
S.spp.	14	32.6
MRSS	4	28.6
MSSS	10	71.4
Total	43	100

Of these 75 isolates, 38 were recovered from the patients admitted in general medical wards, 14 from those admitted in surgical units, and 12 from the patients in pediatrics wards. 11 of the strains were collected from patients from Accident / Emergency and outpatient department. Majority of the organisms from medical and pediatrics wards were isolated from blood. Urine was the second most common source of organisms isolated from medical wards. Pus and blood were the main sources of organisms isolated from surgical wards. Distribution of the organisms regarding their source and location of isolation is given in Table IV. The majority, 22(73.3%) of MRSA were from the medical wards. The same trend was observed for MSSS (40%). MSSA showed equal distribution (33.3%) among each medical and surgical wards. No MRSS could be recovered from medical and surgical wards however, these organisms showed highest distribution 3 (60%) in the paediatric wards.

Table IV: Location and Source (n=75)

Location	Blood	Fluid	Pus	Sputum	Urine	Total
Medicine	24	1	4	4	5	38
Surgery	4	2	6	-	2	14
Paeds	11	-	1	-	-	12
A/E Outdoor)	4	-	5	-	2	11
Total	43	3	16	4	9	75

Diagnosis was not available for 17 (22.7%) of the patients. Among rest of the 58 patients the largest group, n = 21 (36.2%) belonged to general medicine related disease group, n = 16 (27.6%) belonged to nephrology related disease group, n = 13 (22.4%) belonged to general surgery related disease group while n = 8 (13.8.5%) were from hepatology related disease group. The overall distribution of organisms of each group isolated from various disease groups of patients is given in Table V.

Table V: Isolates and Disease groups (n=75)

Isolate	Med	Renal	Surg	Liver	NA	Total
MRSA	6	13	2	4	5	30
MSSA	10	3	9	2	6	30
MRSS	1	-	--	1	3	5
MSSS	4	-	2	1	3	10
Total	21	16	13	8	17	75

NA: Not available

Nephrology related disease group comprised urinary tract infections (UTI), chronic renal failure, acute renal failure, diabetic nephropathy, end stage renal disease, patients on continuous ambulatory peritoneal dialysis and patients on haemodialysis. Patients either having only one, or combinations of any, of these diseases were all included in this group. Hepatology related disease group comprised chronic liver failure, acute liver failure, acute on chronic liver failure, liver cirrhosis, and hepatitis C+ cases. Patients having any of these diseases alone or in combination were all included in this group. General surgery group of diseases comprised soft tissue infections (pre- and post-operative), fistulae, osteomyelitis, fracture bones, diabetic carbuncle, and abscesses. General medicine group of patients included pyrexia of unknown origin (PUO), sepsis, respiratory tract infections, pulmonary kochs, empyema lung, myocarditis, either alone or in combination. All disease groups had a significant proportion of diabetic population among these patients. MRSA were most frequently isolated from nephrology related disease group patients, while MSSA was most commonly found in surgery related disease group of patients.

Antimicrobial sensitivities were performed according to National Committee for Clinical Laboratory Standards[13]. The inhibition zone diameters of vancomycin for all the staphylococci (n=75) ranged from 15 mm to 27 mm. The mean and standard deviation values were 17.4 and 3.2 respectively. The maximum number of isolates (30.7%) showed 16 mm diameter. The inhibition zone diameters of teicoplanin for all the staphylococci (n=75) ranged from 14 mm to 29 mm. The mean and standard deviation values were 17.2 and 4.1 respectively. The maximum number of isolates (26.7%) showed inhibition zone diameter of 16 mm. For staphylococci the breakpoints of sensitivity of vancomycin and teicoplanin were ≥15mm and ≥14mm respectively. All the staphylococci (n=75) were sensitive to both of these antibiotics (Table VI). No statistical difference could be observed for further analysis. Comparison of zone diameters among different groups of staphylococci is also given in Table VI.

Table VI: Glycopeptide Sensitivity in Staphylococci (n=75)

Zone Diameter	Vancomycin	Teicoplanin
Staphylococci (n=75)		
Range	15-27	14-29
Mean±SD	17.4±3.2	17.2±4.1
Sensitive	75 (100%)	75 (100%)
MRSA (n=30)		
Range	15-27	14-29
Mean±SD	17.5±3.2	17.1±4.1
MSSA (n=30)		
Range	15-26	14-28
Mean±SD	17.2±2.8	16.9±3.4
MRSS (n=5)		
Range	16-19	14-20
Mean±SD	16.1±3.5	15.5±3.3
MSSS (n=10)		
Range	16-22	15-22
Mean±SD	16.8±3.7	16.8±4.2

SD: Standard Deviation

Discussion:

This study shows that, in our hospital, there is as yet no significant high-level resistance to glycopeptides in staphylococci – the key gram positive organism involved in infections.

As *S. aureus* and CoNS are the most frequently implicated organisms in gram-positive infections,[5,22] only these were included in our study. *S. aureus* alone accounted for 80% of all the isolates collected in this study. This finding implicates *S. aureus* as a very common cause of gram positive infections in our hospital. *S. aureus* was also the frequent organism isolated from blood. CoNS ranked second in frequency among organisms isolated from blood, however, they accounted for only 20% of all the isolates in this study.

Glycopeptide resistance in clinical isolates of *S. aureus* is rare and, until recently, clinical isolates of *S. aureus* have only expressed intermediate level resistance to teicoplanin.[23,24] The first strain of *S. aureus* (Mu50) to be reported as having reduced susceptibility to vancomycin was isolated in Japan.[9] Subsequently, reports of clinical isolates with intermediate susceptibility to vancomycin have been received from North America[8] and France.[25] Vancomycin resistance in CoNS is also rare from a clinical standpoint but is most appreciated in the biofilm environment on the surface of medical devices[15].

No vancomycin-resistant strains were observed among 60 *S. aureus* and 15 CoNS in our study. This finding is comparable to other studies mentioned above and implicates that vancomycin resistance is rare among clinical isolates of *S. aureus* and CoNS in our hospital.

NCCLS disk diffusion method can detect high-level vancomycin resistance but does not differentiate *Staphylococcus* strains with reduced susceptibility to vancomycin (VISA) (MICs 4 to 8 µg/ml) from susceptible strains (VSSA) (MIC range 0.5 to 2 µg/ml).[10,23] However, VISA are detected by most MIC methods, including Etest and agar screening plates with brain heart infusion agar or Mueller-Hinton agar containing 6 or 5 mg/L vancomycin, respectively [16,26].

While reports of the isolation of VISA are rare, strains of heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hetero-VISA) are relatively common in Japan[11] and have also been reported from the UK,[27] Spain[28] and Germany.[29] Laboratory detection of hetero-VISA is even more difficult than VISA as they exhibit sensitive MIC range for vancomycin. Detection methods of hetero-VISA have been devised on the basis of interaction of methicillin and vancomycin against them. These methods can also detect VISA.[12,30]

We could not detect any glycopeptide-resistant, -intermediate or heterogeneously-intermediate S. *aureus* or CoNS strain. It might be possible that staphylococcal strains with intermediate glycopeptide resistance or hetero resistance are present in our hospital but could not be detected due to inability of the disk diffusion antimicrobial sensitivity testing to detect such strains. Whether such strains are circulating in Pakistan remains to be established. At present, since the exact mechanism of vancomycin resistance in *S. aureus* has not been determined and very little epidemiological data is available, the differences observed may generally be ascribed to the patterns of antibiotic usage.

Conclusion:

We detected no high-level resistance to glycopeptides in staphylococci in our hospital, however, 50% of the S. aureus isolates were methicillin resistant. It is, on the other hand, possible that some heterogeneous intermediate- or low-level resistance to glycopeptides exists in these organisms but could not be detected due to limitations of the disk diffusion method. The findings necessitate strict surveillance of these organisms, institution of effective infection control policies and judicious use of antibiotics. Further studies are needed to find out glycopeptide resistance among staphylococci in Pakistan.

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