In Vitro Activity of Glycopeptides against Clinical Isolates of Enterococci from a Tertiary Care Hospital

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Objective: The objective of this study was to establish the prevalence of glycopeptide (vancomycin and teicoplanin) resistance among clinical isolates of enterococci in Shaikh Zayed Hospital, Lahore, and comparison of antimicrobial sensitivities of vancomycin and teicoplanin among these isolates. **Design:** A comparative analytical study. **Place of study:** This study was conducted in the Department of Microbiology, Federal Postgraduate Medical Institute, Shaikh Zayed Hospital, Lahore. **Materials and methods:** 60 (Sixty) enterococci isolates were collected from clinical specimens received in the laboratory. Identification of these bacteria was done utilizing standard laboratory operating procedures. Their sensitivity to glycopeptide antibiotics was tested by disk diffusion method in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. **Results:** Results show that all enterococci were sensitive to teicoplanin. However, among these isolates 1.7% resistance to vancomycin was detected. **Conclusion:** The results indicate that resistance to glycopeptide antibiotics in the test organisms is low in our hospital. The presence of vancomycin resistance in 1.7% clinical enterococcal isolates necessitates strict surveillance of these organisms, institution of effective infection control policies and judicious use of antibiotics.

Key words: Glycopeptides, Vancomycin, Teicoplanin. Glycopeptide resistance

Infectious diseases remain the leading cause of death worldwide, putting a heavy burden on economy[1]. The incidence of serious bacterial infections is increasing despite remarkable advances in antibiotic chemotherapy.[2] Instead of witnessing the disappearance of bacterial diseases, however, we are now experiencing a resurgence of them, both in hospital and community settings. Furthermore, the bacterial pathogens have become increasingly resistant to a variety of antibiotics[3]. The antibiotic era, barely 60 years old, is currently threatened by the selection of drug-resistant organisms[4].

Gram-positive cocci are one of the major human pathogens worldwide.[5] Antimicrobial resistance in these bacteria has achieved its greatest prominence over the past two decades.[6] Increasing incidence of infections caused by gram-positive bacteria with acquired multidrug resistance is a matter of serious concern[2].

Enterococci are major nosocomial pathogens and their resistance to antibiotics is increasing. Moreover, they may be a reservoir for resistance genes for other gram-positive organisms, including *Staphylococcus aureus*.[7] The most frequent infections caused by enterococci are urinary tract infections. The second most frequent infections are intraabdominal and pelvic sepsis and surgical wound infections. The third most frequent infections are bacteremias, including both primary bacteremias that are presumably from a source in the gastrointestinal tract and bacteremias that are secondary to urinary tract and intraabdominal infections or the use of intravascular devices.

Enterococci are naturally resistant or only moderately susceptible to some antibacterials, e.g. cephalosporins and aminoglycosides, which are active against other gram-positive bacteria[8]. The glycopeptide antibiotics (vancomycin and teicoplanin) are used to treat serious enterococcal infections[9] due to the increasing incidence of resistance in enterococci[10].

Since the first reports of vancomycin-resistant enterococci (VRE) in 1986 in Europe[11,12], their presence has increasingly been detected throughout the world.[13,14] In parallel with their increasing resistance to antibiotics, enterococci have emerged as a major cause of nosocomial infections[7,15,16] Vancomycin-resistant enterococci (VRE) are now the second most common cause of hospital-acquired infections.[13] Once established in the hospital environment, the frequent resistance of VRE to multiple antibiotics makes it difficult or impossible to avoid further selective pressure in their favor[15].

Vancomycin was introduced in Pakistan more than a decade ago, however, it has come into significant clinical use only in the last five years or so (personal communications, Eli Lilly Pakistan Limited). Teicoplanin was launched in 2000 (personal communications, Aventis Pharma Pakistan Limited). In the scenario of prevalent glycopeptide resistance in enterococci worldwide, it was important to evaluate sensitivity of these Antimicrobial drugs against these organisms in Pakistan where no such studies had been conducted. This study looked into the in vitro activity and comparison of glycopeptide antibiotics against enterococci in Shaikh Zayed Hospital Lahore.

Materials and methods:

60 (Sixty) enterococcal bacterial strains 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 *Enterococcus* 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 were excluded from the study. An isolate previously obtained from the same patient was also 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, 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-negative gram-positive cocci showing growth on MacConkey agar (Oxoid) were tested for ability to hydrolyze (bile-) esculin (Enterococcocel agar, BBL). The strains showing positive result were classed as enterococci.[17-19]

Enterococcal clinical isolates collected in the laboratory were stored in TSB (Oxoid) containing 15% (v/v) glycero[17,20]. Five to ten 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[20]. 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 enterococci the breakpoints for 'sensitive' were ≥17 mm and ≥14 mm for vancomycin and teicoplanin respectively. *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[20].

Results:

Sixty (n=60) isolates belonging to *Enterococcus* genus were collected. The majority, 37 (61.7%) of the organisms were isolated from urine while 14 (23.3%) were isolated from blood, 6(10%) from sputum and 3(5%) from fluids (Table I). Distribution of organisms among male and female patients (Table I). 53.3% of isolates were recovered from male while 46.7% from female patients. However the frequency of isolates from urine was higher in females.

Table I: Isolates and Source (n=60)

Source	Male	Female	Total (%)
Urine	17	20	37(61.7%)
Blood	8	6	14(23.3%)
Sputum	5	1	6(10%)
Fluid	2	1	3(5%)
Total	32(53.3%)	28(46.7%)	60(100%)

Of these 60 isolates, 36 were recovered from the patients admitted in general medical wards, 7 from those admitted in surgical units, and 6 from the patients in pediatrics wards. 11 of the strains were collected from patients from Accident / Emergency and outpatient department. Majority of the organisms were isolated from urine. Blood was the second most common source of organisms (Table II).

Table II: Location and Source (n=60)

Location	S O U R C E				Total
	Urine	Blood	Sputum	Fluid	
Medicine	23	6	6	1	36
A/E (Outdoor)	11	-	-	-	11
Surgery	2	3	-	2	07
Paeds	1	5	-	-	06
Total	37	14	6	3	60

Diagnosis was available for 56 patients among whom the largest group, n = 26 (43.3%) belonged to nephrology related disease group, n = 14 (23.3%) belonged to general medicine related disease group, n = 9 (15%) belonged to hepatology related disease group while n = 7 (11.7%) were from general surgery related disease group. Diagnosis was not available for rest, n=4 (6.7%), of the patients. The overall distribution of organisms isolated from various disease groups of patients (Table III).

Nephrology related disease group included patients with, 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, either alone or in combination. 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.

Table III: Disease groups (n=60)

Disease Groups	=n	%age
Renal-related	26	43.3
Medicine-related	14	23.3
Liver-related	9	15
Surgery-related	7	11.7
NA	4	6.7

NA: Not available

Antimicrobial sensitivities were performed according to National Committee for Clinical Laboratory Standards.[20] The inhibition zone diameters of vancomycin for all the enterococci (n=60) ranged from 14 mm-22mm. The mean value was 17.92 and standard deviation was 1.34. Majority of the isolates, 19(31.7%) showed inhibition zone diameter of 17mm. The inhibition zone diameters of teicoplanin for all the enterococci (n=60) ranged from 15mm-20mm. The mean value was 16.75 and standard deviation was 1.1. The maximum number of isolates, 22(36.7%) showed inhibition zone diameter of 16mm.

For enterococci the breakpoints for 'sensitive' were ≥17mm and ≥14mm for vancomycin and teicoplanin respectively. Out of 60 strains, 98.3% demonstrated *sensitivity to vancomycin*, while 1(1.7%) strain was *resistant to vancomycin* with an inhibition zone diameter of 14mm. However, sensitivity to teicoplanin was 100% (Table IV).

Table IV: Glycopeptide Sensitivity in Enterococci (n=60)

Zone Diameter	Vancomycin	Teicoplanin
Range	14-22	15-20
Mean±SD	17.92±1.34	16.75±1.1
Sensitive	59 (98.3%)	60 (100%)
Intermediate	0	0
Resistant	1 (1.7%)	0

SD: Standard Deviation

Discussion:

In our study the majority, n = 37 (61.7%) of the organisms were isolated from urine while n = 14 (23.3%) were isolated from blood. These results are comparable to the findings that the most frequent infections caused by enterococci are urinary tract infections. The second most frequent infections are intraabdominal and pelvic sepsis and surgical wound infections which can frequently give rise to bacteremias.[2]

Enterococci are one of the most frequently implicated organisms in gram-positive infections. In our study 1.7% of the enterococcal isolates demonstrated resistance to vancomycin. The rate of vancomycin resistance in clinical isolates of enterococci has been reported 1.2% in The Aga Khan University Hospital Karachi (Dr. Rumina Hassan, Personal communications) and 2% in Shoukat Khanum Memorial Hospital Lahore (Dr. Faisal Sultan, Personal communication). In the background mentioned above, we can assume that this much resistance is prevalent in our hospital.

Overall incidence of vancomycin resistance in enterococci is increasing worldwide, however there are wide variations in the prevalence of VRE in different parts of the world. For example, one study quotes for approximately 18% of U.S. isolates to be resistant to vancomycin versus 0% of Canadian isolates.[22] Our finding of 1.7% resistance to vancomycin in enterococci is comparable to results from other low prevalence areas in the world.

Glycopeptide-resistant enterococci have different mechanisms of resistance with expression of diverse phenotypes.[23] Vancomycin-resistant enterococci produce modified precursors that terminate in either D-alanyl-D-lactate (D-ala-D-lac) or D-alanyl-D-serine (D-ala-D-ser), which have a much lower affinity for glycopeptides than do unmodified precursors.[24] The genetic basis for resistance lies in genes whose products have homology to the bacterial D-ala-D-ala ligases, encoded by *ddl* genes, which produce the dipeptide target for glycopeptide antibiotics.[25] High-level vancomycin resistance is conferred either by the transferable, inducible VanA or VanB D-ala-D-lac ligases or by the nontransferable, constitutive VanD D-ala-D-lac ligase. However, VanB phenotype is sensitive to teicoplanin whereas VanA and VanC phenotypes are resistant to teicoplanin as well.[25,26] Low-level vancomycin resistance is conferred by VanC or VanE phenotypes. These biochemically and phenotypically similar phenotypes demonstrate intrinsic low-level resistance to vancomycin (MICs 4 to 32µg/ml) and susceptibility to teicoplanin.[15,23,27,28] Disk diffusion method of antimicrobial sensitivity testing is 100% sensitive for the detection of high-level vancomycin resistance in enterococci. For VanB, the sensitivity of disk diffusion method is 93%.[29] As the vancomycin-resistant isolate detected in our study was sensitive to teicoplanin, it can be implicated that the isolate belongs to VanB phenotype of glycopeptide resistance.

Disk diffusion method of antimicrobial sensitivity testing, however, does not effectively detect all the enterococci strains with reduced sensitivity to vancomycin and certain phenotypes (VanC or VanE).[29,30] It is possible that such strains exist in our hospital but could not be identified due to the limitations of the disk diffusion method. In the light of these findings it can be implicated

that the true resistance to glycopeptide antibiotics may be higher than the one we report in our study.

Conclusion:

One (1.7%) of the enterococci tested vancomycin-resistant. The strain was sensitive to teicoplanin, indicating VanB type of resistance. Less frequent use of vancomycin in our hospital may be a reason for this low frequency of resistant strains. It is, however, possible that some intermediate- or low-level resistance to glycopeptides exists but could not be detected due to limitations of the disk diffusion method. True resistance to glycopeptide antibiotics may, therefore, be higher than the one we report in our study. 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 enterococci in Pakistan.

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