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Vancomycin Resistant Enterococcal Infections in Tertiary Care Hospitals of Islamabad and Rawalpindi, Pakistan

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Abstract.- Vancomycin resistant enterococci (VRE) causes health-care related infections such as endocarditis, meningitis, bacteremia, Septicemia and gastrointestinal tract infections in immunocompromised patients. VRE are emerging therapeutic dilemma due to high resistance against commonly used antibiotics including vancomycin. The present study was conducted to investigate the prevalence of VRE in clinical samples isolated from three tertiary care hospitals in Islamabad and Rawalpindi. A total of 106 enterococci *(Enterococcus faecalis*, 52; *Enterococcus faecium*, 54) were isolated during April-September 2009. Out of 106, 54 (50.94%) isolates were resistant to vancomycin as determined by disc diffusion test and culturing on ChromID VRE agar. Maximum and minimum resistance was found against cefotaxime (100%) and teicoplanin (18.86%), respectively. The results showed that teicoplanin is the drug of choice as lowest resistance was observed against this antibiotic as compared to other antibiotics. The MIC of VRE isolates ranged between >4mg/L and ≥512 mg/L for cefotaxime, ciprofloxacin, erythromycin, doxycycline and vancomycin. The high rate of VRE isolation from clinical samples and their resistance to multiple antibiotics suggests a rapid spread of resistance among Enterococci alongwith an emerging shift in VRE distribution.

Key words: *Enterococcus faecalis*, *Enterococcus faecium*, vancomycin-resistant enterococci, minimal inhibitory concentration, vancomycin.

INTRODUCTION

Multi-drug resistant bacterial infections have presented the medical community with an increasing therapeutic dilemma. Of these bacteria, vancomycin-resistant enterococci (VRE) have been paid a particular attention (Padiglione *et al*., 2003). VRE are the third most common cause of health-care–associated infections, despite the fact that they are generally considered to be indolent pathogens (CDC, 1999). Among immunosuppressed or critically ill patients, enterococcal infections are often severe (Patel, 2003). Although, more than one dozen species of *Enterococcus* have been identified, *Enterococcus faecalis* and *Enterococcus faecium* account for approximately 85–90% and 5–10% of human enterococcal infections, respectively (Gold, 2001).

VRE were first identified as hospital-associated pathogens in Europe during the mid-1980s, and have rapidly disseminated worldwide (Leclercq *et al.*, 1988; Uttley *et al.*, 1989). Its occurrence has progressively been increased globally (Deshpande *et al*., 2007). According to a CDC report, 29% of the enterococcal infections in intensive care units (ICUs) were caused by vancomycin- resistant isolates, while 25% infections in non-ICU patients (CDC, 2006). VRE have spread rapidly throughout the world and have become a significant infection control problem for many hospitals (Huang *el al*., 2007).

The intrinsic resistance of enterococci to many antibiotics complicates therapy and the increasing occurrence of acquired resistance aggravates the treatment problems. *Enterococci* are innately resistant to most antibiotics, including; cephalosporins, penicillins, clindamycin and

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trimethoprim (Mascini and Bonten, 2005). Vancomycin is the drug of choice for treating enterococcal infections, so the emergence of vancomycin-resistant strains is of serious clinical concern. There has been an alarming worldwide increase in the rate of infection by VRE in the last 15 years (Murray, 2000).

Risk factors for VRE colonization and infection include; having an underlying co-morbid condition such as diabetes; renal failure or malignancy; prolonged length of hospital stay, particularly with receipt of broad-spectrum antibiotics such as cephalosporins and vancomycin; having an indwelling invasive device or an invasive procedure; and close proximity to another VRE-colonized or -infected patient (Vergis *et al*., 2001).

Enterococci cause a wide range of infections that includes: endocarditis, septicemia, urinary tract infections, intra-abdominal and wound infections as well as infections of indwelling lines. Their significance as the third leading cause of nosocomial infections emphasizes the importance of understanding the pathogenesis of these organisms such that more efficient prevention and treatment methods can be developed. This is especially relevant as treatment options currently are extremely limited (Koch *et al*., 2004).

There has been a rapid increase in the incidence of MRSA infections in Pakistan and this has resulted in increasing use of glycopeptides (Hafiz *et al*., 2002). Recent administration of vancomycin, use of multiple antibiotics, prolonged hospital stay and immunosuppression are all recognized as risk factors for the development of vancomycin resistance in the enterococci (Cetinkaya *et al*., 2000). First case of VRE in Pakistan was reported in 2002 from Karachi (Khan *et al*., 2002).

The first guidelines for the control of VRE in hospitals were published in 1994 by the CDC Hospital Infection Control Practices Advisory Committee (HICPAC) (Lancaster, 1994). Despite the application of infection control protocols, VRE is still endemic in many hospitals and its incidence is increasing around the world (Pearman, 2004).

The aim of this study was to investigate the prevalence of vancomycin resistant enterococci in the clinical samples from patients admitted in tertiary care hospitals of Islamabad and Rawalpindi.

MATERIALS AND METHODS

Sampling

A total of 133 Gram positive cocci were isolated on blood agar from different specimens, such as; urine, blood, pus, tissues, surgical sites, etc., in a period of 6 months (April – September, 2012). Samples were collected from the patients admitted in different wards (*i.e.*, Medical and Surgical wards) of three different hospitals; Pakistan Institute of Medical Sciences (PIMS), Shifa International Hospital, Islamabad, and Holy Family Hospital, Rawalpindi.

Isolation and identification of Enterococcus *species*

Enterococci were isolated by culturing the Gram-positive cocci from blood agar plates on the Chromocult® Enterococci Agar (Merck) and incubated for 24 h at 35-37°C. The species of enterococci *i.e.*, *E. faecalis* and *E. faecium* were identified through biochemical tests. Three tests were performed to identify the species *i.e.*, arabinose and sorbitol fermentation and growth at 4°C. Arabinose and sorbitol fermentation tests were performed by adding 0.5% of the sugars to the nutrient broth and using phenol red as indicator. *E. faecalis* can ferment sorbitol only while cannot grow at 4°C, and *vice versa* in case *E. faecium*, it can ferment arabinose instead of sorbitol.

Isolation of vancomycin resistant enterococci

The *Enterococcus* species were then inoculated on the ChromID™ VRE (Biomerieux) medium and incubated for 24 h at 37°C. ChromID VRE has been designed for the screening of VRE. After 24 h of incubation, the results were noted on the basis of colour; the *E. faecalis* showed bluish green colonies while violet colonies of *E. faecium* were observed.

Antibiotic susceptibility testing

Isolates were then checked for their susceptibility towards different antibiotics. A total of 14 antibiotic discs (ampicillin 25µg, cefotaxime 30µg, cefpirome 30µg, chloramphenicol 30µg, ciprofloxacin 5µg, clindamycin 2µg, doxycycline 30µg, erythromycin 15µg, gentamycin 10µg, levofloxacin 5µg, linezolid 30µg, sulbactam/

cefoperazone 105 µg, teicoplanin 30 µg and vancomycin 30 µg) were tested against 106 *Enterococcus* isolates to check whether these are sensitive, intermediate or resistant to these antibiotics. Antimicrobial susceptibility testing was performed on Mueller Hinton agar by modified Kirby-Bauer disc diffusion method.

Overnight fresh cultures were used to make lawns on Mueller-Hinton agar (MHA) (oxoid, UK). The inoculum was prepared in normal saline by emulsifying the enterococcus colonies of isolated colonies selected from an 18 to 24 h agar plate (a nonselective medium, such as blood agar). The suspension was adjusted to match the 0.5 McFarland turbidity standard (1.5 $x10^8$ CFU/ml), using saline and a vortex mixer. A sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removed excess inoculum from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The plate was allowed to dry for 5 min. The antibiotics discs were dispensed onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. The discs were placed, not closer than 24 mm from center to center. About 12 discs were placed on one 150 mm plate or more than 7discs on a 90 mm plate using modified Kirby-Bauer method. The plates were incubated at 35°C for 16 to 18 h in incubator. Zones of inhibition in millimeters were measured, recorded and the isolates were classified as resistant, intermediate, sensitive and according to clinical laboratory standard institutes criteria (CLSI, 2012).

Minimum inhibitory concentration (MIC)

Antimicrobial sensitivity of five antibiotics (ciprofloxacin, cefotaxime, doxycycline, erythromycin and vancomycin) was checked by determining MIC against VRE strains using agar dilution method. Standard powders of antibiotics were supplied by Oxoid, Basingstok, Birmingham, UK, and were used to make stock solutions.

Stock solutions from the antibiotics powders were prepared by using the formula: 1000/P x V x C = W. Here, P represents for potency given by the manufacturer (µg/mg), V is the volume required (ml), C final concentration of the solution (multiples of 1000) (mg/l), and W represents weight of antibiotic in mg to be dissolved in volume V (ml). Stock solutions were prepared by adding known quantity of antibiotic powder in respected sterile diluents. Antibiotic dilution range of 0.25-1024 µg/ml in flasks according to the antibiotic breakpoints for that particular species. In the case of control, no antibiotic was added to the flask.

Preparation of agar dilution plates

Approximately 20 ml of cooled molten agar was poured into each flask containing different concentration of antibiotic solution. The media was mixed well and poured into the 90 mm petri dish. Allow the agar to set and then dry the surface of plates and used immediately.

Preparation of inoculum

A bacterial suspension was prepared by mixing four colonies of *Enterococcus* sp. and were transferred to nutrient broth. Broth was then placed in incubator shaker at 35-36ºC until the visible turbidity was equal to or greater than the 0.5 McFarland standard (1.5 $x10^8$ CFU/ml). It was used within 30 minutes of preparation.

Statistical analysis

All the data was entered and analyzed by calculating percentages using SPSS software (SPSS Inc. USA).

RESULTS

Identification of enterococci

Gram-positive cocci were identified as enterococci by their growth on Chromocult® Enterococci Agar (Merck). Enterococci formed pinhead red colonies on Chromocult® Enterococci agar with diameter of about 0.5 to 2 mm. Out of 133 isolates, 106 were identified as enterococci. Among these 106 enterococci, 65 were from Shifa International Hospital, 8 from Holy Family Hospital

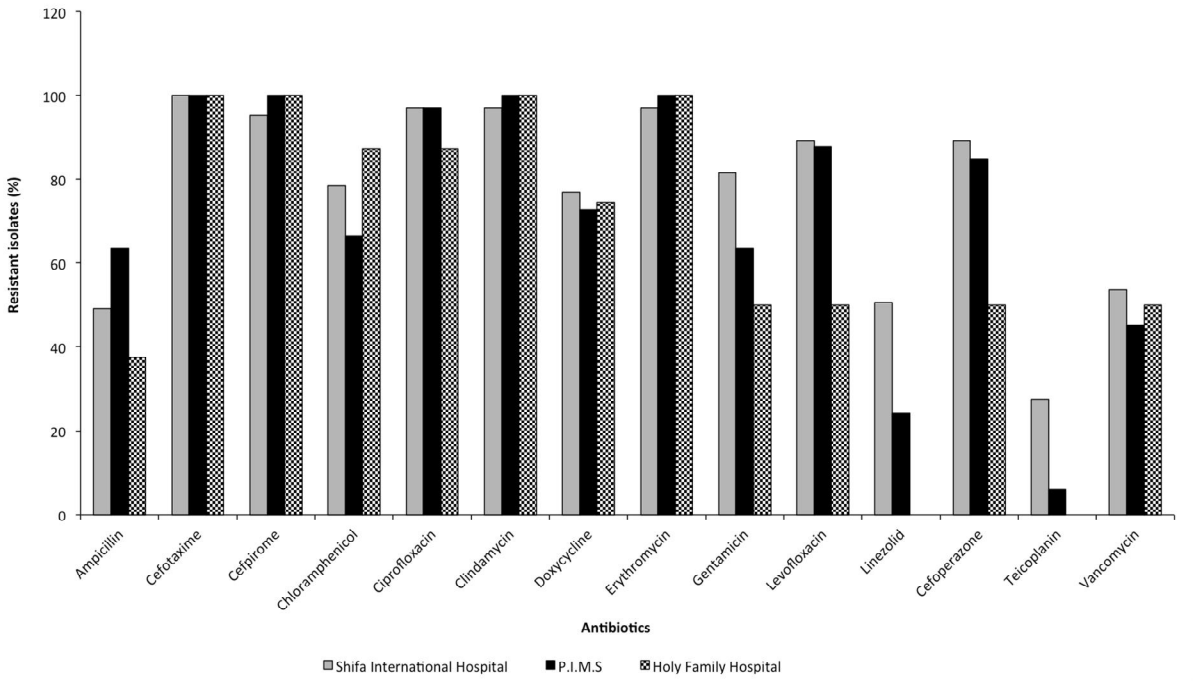

Fig. 1. Antibiotic resistance pattern of all isolates collected from three hospitals.

and 33 from PIMS. Enterococci were mainly recovered from urine 59 (55.66%), and pus 16 (15.09%) specimens, followed by blood 14 (13.20%), catheter tip 08 (7.54%) wound 08 (7.54%) and tissues 01 (0.94%), respectively.

Out of 106 isolates, 52 (49.05%) were identified as *Enterococcus faecalis*, while 54 (50.94%) were *Enterococcus faecium*. Out of total *Enterococcus faecalis*, 28 (43.07%) were obtained from Shifa International, 06 (75%) from Holy Family Hospital and 18 (54.54%) were from PIMS. Similarly, out of total *Enterococcus faecium*, 37 (56.92%) were from Shifa International Hospital, 02 (25%) from Holy Family Hospital and 15 (45.45%) from PIMS.

Antibiotic susceptibility

Out of 106 isolates, 78 (73.58%) were resistant to gentamicin, 106 (100%) to cefotaxime, 103 (97.16%) to cefpirome, 102 (96.20%) to ciprofloxacin, 91 (85.84%) to levofloxacin, 54 (50.94%) to vancomycin, 20 (18.86%) to teicoplanin, 104 (98.11%) to clindamycin, 104 (98.11%) to erythromycin, 41 (38.67%) to linezolid, 56 (52.83%) to ampicillin, 79 (74.52%) to doxycycline, 80 (75.47%) to chloramphenicol and to 90 (84.90%) sulbactam/cefoperazone (Fig. 1).

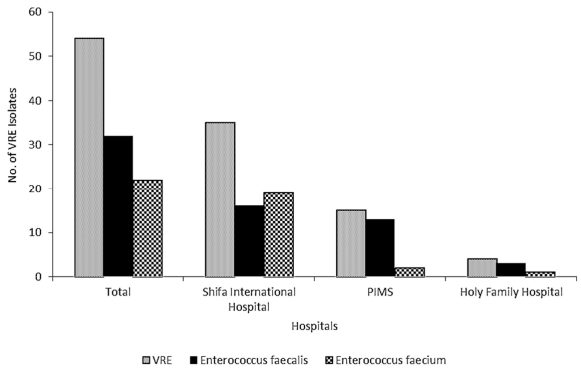

Fig. 2. Frequency of vancomycin resistant enterococci (VRE) in three hospitals.

Frequency of VRE

The number and percentage of VRE and vancomycin sensitive enterococci (VSE) in different hospitals were determined by the antibiotic disc diffusion test and by culturing on ChromID VRE medium (Fig.2). About 54 (50.94%) isolates of enterococci were found resistant to vancomycin, 32

Table I.- Antibiotic susceptibility profile of 52 VSE and 54 VRE isolates against different antibiotics.

	Shifa (n=65)		PIMS (n=33)		Holy Family (n=8)		Total (n=106)	
	VSE (n=30)	VRE (n=35)	VSE (n=18)	VRE (n=15)	VSE (n=4)	VRE (n=4)	VSE (n=52)	VRE (n=54)
AMP	6	27	2	10	1	4	9	41
C	9	5	8	3	0	1	17	9
CIP	2	0	1	0	0	1	3	1
CN	4	8	7	5	3	1	14	14
CPO	2	1	0	0	0	0	2	1
CTX	0	0	0	0	0	0	0	0
DA	2	0	0	0	0	0	2	0
DO	11	4	7	2	0	3	18	9
E	2	0	0	0	0	0	2	0
LEV	3	4	2	2	0	4	3	10
LZD	24	8	15	10	4	4	43	22
SCF	2	5	1	4	0	4	3	13
TEC	27	20	17	14	4	4	48	38

(59.25%) were *Enterococcus faecalis* and 22 (40.74%) *Enterococcus faecium.* The rest of enterococci isolates were sensitive to vancomycin *i.e.* 49.05%. The activity of different antibiotics against VRE and VSE is given in Table I.

MIC) of antibiotics against VRE strains

MIC of cefotaxime, ciprofloxacin, doxycycline, erythromycin and vancomycin against VRE strains was checked. Against cefotaxime, 05 (9.25%) VRE strains showed MIC at 64 mg/L, 02 (3.70%) strains at 128 mg/L, 06 (11.11%) at 256 mg/L and 41 (75.92%) strains showed MIC at ≥512mg/L. In case of erythromycin, 01 (1.85%) strain showed MIC at 0.5 mg/L, 03 (5.55%) at 2 mg/L, 05 (9.25%) at 4 mg/L, 02 (3.70%) at 8 mg/L, 01 (1.85%) at 64 and 128 mg/L each, and 41 (75.92%) strains showed MIC at ≥512 mg/L. MIC for ciprofloxacin was also determined and it was found that 01 (1.85%) strain at 4 mg/L, 10 (18.51%) strains at 8 mg/L, 07 (12.96%) strains at 16 mg/L, 02 (3.70%) strains at 32 mg/L, 07 (12.96%) strains at 64 mg/L, 20 (37.03%) strains at 128 mg/L and 07 (12.96%) strains showed MIC at ≥256 mg/L. MIC values against doxycycline were from 4 mg/L to 128 mg/L. MIC for 03 (5.55%) strains was 4 mg/L, 11 (20.37%) strains at 8 mg/L, 10 (18.51%) strains at 16 mg/L, 15 (27.77%) strains at 32 mg/L, 11 (20.37%) strains at 64 mg/L and 04 (7.40%) strains at 128 mg/L. Vancomycin was also included in the study to determine its MIC against VRE strains. A total of 52 (96.29%) strains showed MIC at >4mg/L, while only 02 (3.70%) strains showed highest MIC at ≥512 mg/L.

DISCUSSION

Acquired resistance against antibiotics is closely related to the amount of drug used, a fact observed ever since these agents were introduced into human and veterinary medicine. However, the rate of development of resistance appears to have accelerated since more than a decade (Saleem *et al*., 2015).

In our study total 106 *Enterococcus* isolates were collected from three different tertiary care hospitals of Rawalpindi and Islamabad, out of which 52 were *Enterococcus faecalis,* while remaining 54 isolates were *Enterococcus faecium.* The results were in concordance with the study of Mohanty and colleagues (2005), who reported that the predominant isolates were *E. faecium* (42.90%) and *E. faecalis* (40.00%), respectively. Akhter *et al.* (2011) reported *E. faecalis* to be the most common, followed by *E. faecium* in the clinical samples. Similarly, more than 90% of *E. faecalis* were reported by Butt and his colleagues (2004) in their report. Sharifi *et al.* (2011) also reported high percentage of *E. faecalis* as compared to *E. faecium.* Most of the enterococci were isolated from urine followed by pus, and blood. Maximum number of enterococci have been reported from urine, blood,

burn wounds and stool samples (Japoni *et al*., 2009; Prakash *et al*., 2005).

VRE were isolated by culturing on ChromID VRE medium and using antibiotic discs. Delmas *et al*. (2007) also used ChromID medium for the evaluation of VRE. The overall prevalence of VRE was 54%. The VRE cases were reported in other parts of the world also with different percentages like 35% in Iran (Japoni *et al*., 2009), 9.6% in Israel (Benenson *et al*., 2009), 7.7% in Brazil (Kobayashi *et al*., 2011) and 21% in Serbia (Mihajlović *et al*., 2011). Hospital outbreaks of VRE have been reported extensively in the United States, with a prevalence as high as 47% in some studies (Deshpande *et al*., 2007). In Pakistan, the first VRE was reported in 2002, about 10 vancomycin resistant *E. faecalis* isolates were reported in Agha Khan University Hospital, Karachi (Khan *et al*., 2002). Hospital outbreaks of VRE have been reported extensively in the United States, with a prevalence as high as 47% in some studies (Cetinkaya *et al*., 2000).

The MIC of vancomycin showed that out of 54 VRE strains only two strains were highly resistant to vancomycin *i.e.*, MIC ≥512 mg/L and remaining 52 VRE strains showed their MIC at >4 mg/L. Enterococci isolates were found to be resistant to multiple groups of antibiotics. In our case the resistance rate was high in case of VRE and VSE, against different groups of antibiotics except teicoplanin and linezolid, as indicated by the figure. The association of elevated resistance was more pronounced among VRE isolates against ampicillin, streptomycin and rifampin. Japoni *et al.* (2009) also reported resistance in VRE isolates against ciprofloxacin, amikacin and gentamicin. In our case the resistance rate was higher in case of VRE and VSE, against both ciprofloxacin and levofloxacin as compared to the previous studies (Aleyasin *et al*., 2007; Mascini and Bonten, 2005). The resistance of enterococci to multiple antibiotics is common as it is also observed in other parts of the world (Feizabadi *et al*., 2004; Rudy *et al*., 2004). Teicoplanin and linezolid were found effective against the enterococci isolates. VRE strains were found susceptible to tigecycline and linezolid (Japoni *et al*., 2009). Teicoplanin and linezolid were found effective against enterococci in other parts of the world (Akhter *et al*., 2011; Sharifi *et al*., 2011; Kobayashi *et al*., 2011). About 17.30% of VSE, and 59.25% of the VRE strains, were resistant to linezolid. The high rate of sensitivity than resistivity to linezolid confirm our findings to those by Ballow *et al*. (2002) who demonstrated the wide spectrum of action of this drug in Latin American centers. Khan *et al*. (2002) reported high-level resistance to both glycopeptides with a vancomycin minimum inhibitory concentration greater than 256 mg/L.

CONCLUSIONS

The rate of resistance against vancomycin is a serious threat that necessitates using surveillance studies, infection control and monitoring of antibiotic sensitivity among hospital isolated strains. The prevalence of antibiotic resistance among the studied isolates, their presence together with aminoglycoside resistance calls for regular surveillance of antibacterial susceptibilities to detect the emerging resistance and prevent the establishment and spread of multidrug antibacterial-resistant strains.

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Conflict of interest

There is no conflict of interest and all the authors agreed to submit it to Pakistan Journal of Zoology. The members of ethical committee of our institute have already approved the submission of this manuscript to any journal for publication.

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