In vitro efficacy of linezolid against vancomycin resistant *Enterococci*

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Abstract: Vancomycin–resistance among *Enterococci* has been escalating in recent years in many countries. Linezolid, an oxazolidinone is one of the novel drug that promised effective therapy of infections caused by vancomycin-resistant *Enterococci* (VRE).However, like with most of the previous antimicrobial agents, the clinical benefit of linezolid is being threatened by the emergence of resistant strains of MRSA and vancomycin resistant *enterococci* VRE, being reported in many countries. This study was conducted to establish linezolid susceptibility of VRE isolates by determining the *in vitro* activity of linezolid against vancomycin resistant *Enterococci*, isolated in our setup using fifty VRE isolates. The data collected from this study conclude that the vancomycin resistant *Enterococci* are 100% linezolid susceptible and presently there is no significant resistance against this unique oxazolidinone in our setup.

Keywords: Linezolid, VRE, MIC, Agar Dilution Method, E-Test

INTRODUCTION

Enterococci are organisms owing broad spectrum of intrinsic and acquired antibiotic resistance along with carriage of several virulence genes (Rice *et al.,* 2003). *Enterococci* cause frequent nosocomial infections and are ranked second as etiological organisms of hospital-acquired infections in US hospitals.

Vancomycin had remained the last resort for managing multi-resistant enterococcal infections for more than three decades. Unfortunately, the majority of *Enterococcus faecium* isolates are now found to be resistant to vancomycin, worldwide.

In Europe, the prevalence of vancomycin resistance *Enterococci* has increased enormously, with important regional differences (Brown *et al.,* 2008; Werner *et al.,* 2008). Deshpande *et al.,* (2007) have reported a 13.9% resistance among *Enterococci* against vancomycin in 1993 representing a 20 fold increase from 1989.

Linezolid, a novel drug of the oxazolidinone family, acts as an antimicrobial agent through a mechanism unique to this class. It inhibits the formation of the initiation complex thereby halting bacterial protein synthesis and growth. Approved for clinical use in 2000, linezolid has been successfully used to treat VRE infections (FDA, 2005). However, like with most of the previous antimicrobial agents, the clinical benefit of linezolid is being threatened by the emergence of resistant strains of MRSA and vancomycin-resistant *Enterococci* in many countries. Increasing consumption (Overuse) of linezolid has been identified as possible cause of this rising bacterial resistance suggesting the need for its cautious use as well as performing susceptibility tests and running surveillance programs to detect any decreasing Linezolid susceptibility over time (Schulte *et al.,* 2008).

The purpose of our study was to determine *in vitro* efficacy of linezolid in view of determining any developing resistance/intermediate resistance against linezolid among vancomycin resistant *Enterococci* isolated from our setup.

Although, *Enterococci* are normal inhabitants of human intestines, they may result in serious opportunistic infections particularly in those with compromised host defense mechanisms. Enterococci can cause various infections like urinary tract infections, endocarditis, intra-abdominal and pelvic infections, wound infections meningitis, respiratory tract infections, neonatal sepsis and osteomyelitis.

Enterococci have remarkable ability to develop resistance to antimicrobials into clinical use. Till early 1980s, vancomycin was considered as the last line of defense against the *Enterococci* that were resistant to all other antibiotics, but after that era, there was emergence of resistance against vancomycin among *Enterococci* (Cetinkaya *et al.,* 2000). Approximately 30% of all enterococcal infections are now caused by vancomycin-resistant strains. Particularly virulent strains of *Enterococcus* that are resistant to vancomycin (VRE) have emerged and resulted in life-threatening nosocomial infections during the last two decades, especially in the US hospitals (Fisher and Phillips, 2009). Sixty percent of such infections are found to be caused by *Enterococcus faecalis* (Kozuszko *et al.,* 2009). To become vancomycin-resistant, vancomycin-sensitive *Enterococci* obtain new DNA either in the form of plasmids or transposons which encode genes to confer vancomycin resistance.

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Table 1: Source of specimen, VRE isolates (N=58) and isolated organism from VRE

Specimen source	VRE isolates (N=58)	Number of isolated species from VRE	
		Enterococcus faecium(n=39)	Enterococcus faecalis(n=19)
Pus	35(60%)	25	10
Urine	12(21%)	05	07
Blood	08(14%)	06	02
Body fluids	3(5%)	03	00

Table 2: Minimum inhibitory concentration of linezolid against VRE (N=58)

MIC (mg/L) Linezolid	0.25	0.50	0.75	1	1.5	2
Total number of Susceptible isolates 58(100%)	01(1.7%)	26(44.8%)	17(29.3%)	08(13.8%)	04(7.0%)	02(3.4%)

Clinicians are then left with limited therapeutic options for effective treatment of VRE infections. Linezolid, together with the recently licensed drugs (e.g. quinupristin-dalfopristin, daptomycin and tigecycline and many other experimental agents), represent an effective response to these multi drug resistant infections. Their unique mechanisms of action, their effective pharmaco dynamics, possible synergism with other compounds (effective against Gram-positive pathogens) and their safe and easy administration (also when compromised patients are of concern), all make them reasonable and effective therapeutic choices against VRE (Manfredi, 2007).

Linezolid is the first completely synthetic new antimicrobial (of oxazolidinone class of drugs), which has been found to be active against most Gram-positive bacteria notably vancomycin-resistant *Enterococci* (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA) (Gilmore *et al.,* 2002). It has a unique mechanism of action of inhibiting the bacterial growth through disruption of their protein synthesis. Owing to its unique mechanism of action, Linezolid was claimed spared of developing any cross resistance at its introduction (Fines and Leclercq 2000; Zurenko *et al.,* 1996), but resistance does appear. In 2001 the first isolate of Linezolid-resistant *Staphylococcus aureus* was identified (Lovering *et al.,* 2009).

In the United States, resistance to linezolid has been monitored since 2004 through a program known as LEADER and is found to be stable and extremely low (Flamm *et al.,* 2012). Another worldwide programme ZAAPS has confirmed high and maintained linezolid activity (Ross *et al.,* 2011).

Linezolid resistance among *Enterococci* usually results from a single point mutation known as *G2576T*. This mutation includes the replacement of a guanine with thymine in a base pair 2576 of genes coding for 23S rRNA. The main objective of this study was to determine *in vitro* efficacy of linezolid against vancomycin resistant *Enterococci*.

MATERIAL AND METHODS

It was a descriptive cross sectional study. The study was conducted at the Department of Microbiology, AFIP, Rawalpindi, Pakistan. The duration of the study was 6 months from August 2012 to January 2013. Fifty eight (58) clinical isolates (*Enterococci*) which were resistant to vancomycin were selected. *Enterococci* resistant to vancomycin were included and repeated samples were excluded.

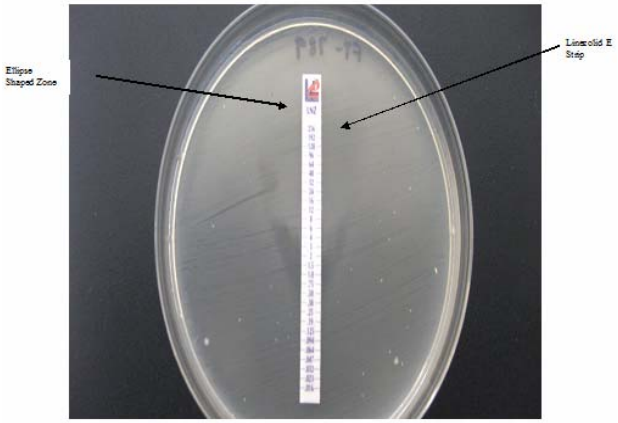

Fig. 1: Ellipse shaped zone of no growth along E- strip of linezolid showing MIC value

Methodology

Bacterial isolates

A total of fifty eight (58) vancomycin resistant isolates were analyzed, including 39 *Enterococcus faecium* and 19 *Enterococcal faecalis* isolates. These strains were isolated from clinical samples of urine, pus, fluids, blood, CSF etc. Specimens were cultured on Blood and Mac Conkey agar plates (CLED agar in case of urine samples).

Colonial & microscopic characteristics

Keeping in mind the specific colonial and microscopic morphology of *Enterococci*, Gram staining of suspected *Enterococci* that had characteristic colonies over agar plates was performed (Duguid, 1996).

Biochemical profile

Enterococci were confirmed by standard laboratory methods (Cowan *et al.,* 2004) including:

I. Catalase test (negative for almost all *Enterococci*)
II. Hydrolysis of bile aesculin (positive for *Enterococci*)
III. Growth in 6.5% NaCl broth (positive for *Enterococci*)
IV. Lancefield grouping (*Enterococci* fall in group D)

Antimicrobial sensitivity

Antimicrobial sensitivity was done by modified Kirby-Bauer disk diffusion method (CLSI 2015) (Oxoid UK) (Kirby *et al.,* 1966). After 24 hrs, zone sizes were measured to find out resistance among *Enterococci* against vancomycin.

Organisms were considered

Sensitive at zone size = ≥17 mm
Intermediate = 15-16 mm
Resistant = ≤14 mm
Enterococci resistant to Vancomycin were selected (zone size ≤14 mm).

Resistance to Vancomycin among *Enterococci* was confirmed by determining MICs of Vancomycin through agar dilution method. Serial dilutions of antibiotics were made to determine the MIC values (CLSI 2015).

All susceptibility test results were assessed after 24hr incubation at 35ºC. Organisms were considered:
(Sensitive = ≤4mg/L, Intermediate =8–16mg/L, Resistant = ≥32mg/L)
MICs of vancomycin for all suspected vancomycin resistant *Enterococci* (VRE) were more than 32 mg/L

API 20 strep

Each vancomycin resistant Enterococcus was reconfirmed as *Enterococcus* species by using API strips (Alper *et al.,* 2004) (Bio Merieux, France).

Preservation of isolates

After dealing the isolates, they were preserved in nutrient glycerol broth at -80^{o}C as per standard protocol (McBryde *et al.,* 2007, Willey *et al.,* 1999).

E-test

E-test is basically related to arraying a concentration gradient of an antibiotic on a polymer strip. (Alper *et al.* 2004).

Procedure

Isolated colonies of vancomycin resistant *Enterococci* were picked and dissolved in 5ml of normal saline to make a 0.5 McFarland solution which was swabbed on Mueller-Hinton agar to make a lawn.

E-strip of linezolid was placed in the center of plate and incubated at 37^{O}C for 18 to 24 hours.

Interpretations/Results

After 24 hours, an ellipse shaped zone of no growth was obtained. Minimum inhibitory concentration (MIC) was read from concentration markings on the E test strip where ellipse met that strip.

Isolates having minimum inhibitory concentration ≥8 mg/L of linezolid were considered resistant, intermediate at 4 mg/L and sensitive at ≤2mg/L (CLSI 2015).

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RESULT

Out of 58 VRE isolates recovered from various clinical specimens, 42 (73%) were from male patients while remaining 16 (27%) were from female patients. Age range of the patients giving isolates of VRE was 6-75years with a mean of 38 years. Maximum number of VRE isolates 35 (60%) were recovered from pus specimens followed by urine 12 (21%), blood 8 (14%) and body fluids 3 (5%). of 58 enterococcal isolates, 39 were *Enterococcus faecium* while only 19 isolates were *Enterococcus faecalis* (Table 1).

All the enterococcal isolates were sensitive to linezolid. The minimum inhibitory concentrations of various isolates ranged from 0.25mg/L to 2mg/L. The highest MIC value for linezolid was 2mg/L shown by only two isolates in this study. Whereas the majority of isolates had MIC's much lower than this value with a mean of 0.75mg/L; detailed summary of results, showing the isolate origin, isolated organism, susceptibility to Linezolid and MIC values are given in table 2.

DISCUSSION

The results of our study agree with many other published data which show that linezolid has maintained excellent *in vitro* activity against multi-drug resistant Gram–positive cocci, including vancomycin resistant *Enterococci* and development of resistance against linezolid is rare despite its increasing use.

The Microbiology Laboratory of Uludag University Medical Faculty Hospital, Turkey conducted a similar study to investigate the *in vitro* susceptibilities of MRSA and VRE clinical isolates to linezolid. They found that all of the isolates were susceptible to linezolid (Efe *et al.,* 2009). In our case MIC for vancomycin resistant *Enterococci* is 0.50-2.0 mg/L.

Another study was conducted in Brazil. The highest MIC detected for this compound in this case was 2µg/mL (Reis *et al.,* 2001) which is exactly the same as found in our study.

The results, although encouraging, do necessitate the need for measures to avoid the progression of resistance against this novel drug at the same time. We also suggest that careful and ongoing monitoring of the *in vitro* effectiveness of linezolid needs to be ensured in order to identify any changes contrary to these projections as soon as possible. Thus, this study presents *in vitro* information, *in vivo* studies are required in our set up to support the clinical use of linezolid against VRE.

CONCLUSION

The results of our study conclude that the isolated strains of vancomycin resistant *Enterococci* in our set up are highly sensitive to linezolid and currently there was no isolate resistant to linezolid.

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