Original Article

Vancomycin resistant enterococci and detection of responsible genes

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ABSTRACT

Objective: Vancomycin-resistant enterococci (VRE) have been increasing especially in hospital acquired infections. The present study was carried out to determine prevalence of VRE in hospitalized patients and detection of their drug resistance genes by molecular technique.

Methodology: The antibiotic susceptibility tests were performed using disk diffusion and E-test. PCR was performed to detect vancomycin resistance genes.

Results: Out of 585 enterococcal isolates, 6 (1%) were positive for VRE. 238 isolates (40%) exhibited resistance to high level gentamicin. Among VRE 50% were identified as *E. faecium* and the entire VRE bacteria posses *van A* gene.

Conclusion: The present study provides the first local data on the prevalence of VRE in Malaysia and would serve as an alert to the clinicians of the emergence of VRE infections and to take appropriate measure to prevent them.

KEY WORDS: Vancomycin resistance, Enterococci, Antibiotic susceptibility, Polymerase chain reaction, Van A gene.

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INTRODUCTION

Vancomycin is an antibiotic that is often used to treat infections caused by enterococci. In some cases, enterococci have become resistant to vancomycin and are called vancomycin-resistant enterococci or VRE.[1] The acquisition of vancomycin resistance by enterococci has seriously affected the treatment and infection control of these organisms. VRE, particularly *E faecium* strains, are frequently resistant to all antibiotics that are effective treatment for vancomycin-susceptible enterococci, which leaves clinicians treating VRE infections with limited therapeutic options.[2]

Since their initial recovery from patients in the United Kingdom and France, VRE have been found in many other countries, including Australia, Belgium, Canada, Denmark, Germany, Italy, Malaysia, The Netherlands, Spain, Sweden and the United States.[3]

National nosocomial infections surveillance system (NNIS)[4] reported that more than 25% of health care-associated enterococcal infections found to be associated with organisms resistant to vancomycin. In Malaysia a confirmed case of vancomycin resistant *Enterococcus* isolated from blood from a young woman with chronic renal failure was first reported in Hospital Kuala Lumpur (HKL).[5] However, later on systematic study has not been performed to determine the prevalence of VRE in the country. Therefore, the present study was undertaken to determine the prevalence of VRE in hospitalized patients and detection of responsible genes.

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METHODOLOGY

Study area and population: The study was undertaken from May 2007 to April 2008 at Hospital Kuala Lumpur (HKL), Malaysia. The hospital consists of 3000 beds and 90 wards which provide services and has been working as a reference centre for the hospitals of other states in the country. A total number of 585 samples were collected from the patients of HKL with different infections during the period.

Sampling and transportation to the laboratory: The samples collected from the patients were: blood, urine, pus, tissue, body fluids, swabs from wound and placenta in appropriate containers. The samples were transported immediately to the Hospital Laboratory of Kuala Lumpar for detection and characterization.

Collection of clinical information: Information on patient's profiles: age, diagnosis, risk factors such as duration of hospitalization (prolonged >2 weeks), catheter use, usage of antibiotics, malignancy, use of steroids were obtained from the medical records.

Identification of enterococcus spp: Enterococci were identified based on standard protocol to study morphology, staining characteristics, biochemical reactions. In addition the bacteria were finally confirmed by Slidex Strepto-Kit; Bio Merieux and API 20Strep kit BioMerieux, France.

Susceptibility testing: Antibiogram of the isolates was determined by disk diffusion method, testing for vancomycin (30µg), teicoplanin (30µg), ampicillin (10µg), high concentration of gentamicin (120µg) and linezolid (30µg). The tests were performed using the methodology recommended by the Clinical and Laboratory Standards Institute.[6]

Disk Diffusion Method: Susceptibility testing of enterococci to ampicillin, high level gentamicin, vancomycin, teicoplanin and linezolid was determined by a Kirby-Bauer disk diffusion method as per CLSI criteria.[6]

E-test of vancomycin: E-test was performed as per the procedure of Clinical and Laboratory Standard Institute (CLSI).[6] Minimum inhibitory concentration (MIC) for vancomycin was determined by E-test and an isolate was considered susceptible to vancomycin if the MIC was observed d" 4µg/ml and resistant if MIC >32 µg/ml.

Control starins: ATCC 29212 and ATCC 51299 were used as sensitive and resistant controls, respectively. The MIC values of vancomycin for the control strains ranges provided by theCLSI.[6]

Molecular detection of vancomycin resistance genes: Vanocomycin-resistance genes were detected by PCR using specific primers as per the procedure of Boyd et al.[7] Briefly, PCR reaction was performed in a 50µl volume consisting of: 10X PCR buffer, 3.5 mM $MgCl_2$, 0.5 µl of each primer, 2.5 µl Taq DNA polymerase, 0.2 µl dNTP and 3µl of DNA template (10 µg/mL). PCR conditions consisted of a pre-denaturation step at 94°C for 5 min, followed by 30 cycles of 45 sec denaturation at 94°C, 45 sec annealing at 54°C and 45 sec extension at 72°C. A final extension step was performed at 72°C for 5 minutes. Amplified products were analyzed by electrophoresis on 1.5% agarose gel.

Statistical analysis: All statistical analyses were carried out using SPSS version 12.0.

Ethics Committee approval: The research work was approved by Ethics committee after finalization of the project proposal.

RESULTS

A total number of 585 isolates of Enterococci were identified at HKL during 12 month study period. Isolates from various clinical specimens in which majority were from urine, followed by blood, pus, tissue and other non-sterile specimen (placental swab, bile, vaginal swab). Out of them six (1%) were confirmed as vancomycin-resistant *enterococci*. These 6 VRE isolates were further identified as *E. faceium* (4/6), *E. faecalis* (1/6) and *E. avium* (1/6). Polymerase chain reaction for all this VRE isolates showed presence of *vanA* gene.

Table-I shows that a total number of 585 enterococci isolates tested by disk diffusion method 99% were susceptible to Vancomycin and 1% was found to be resistant. However, all the isolates were sus-

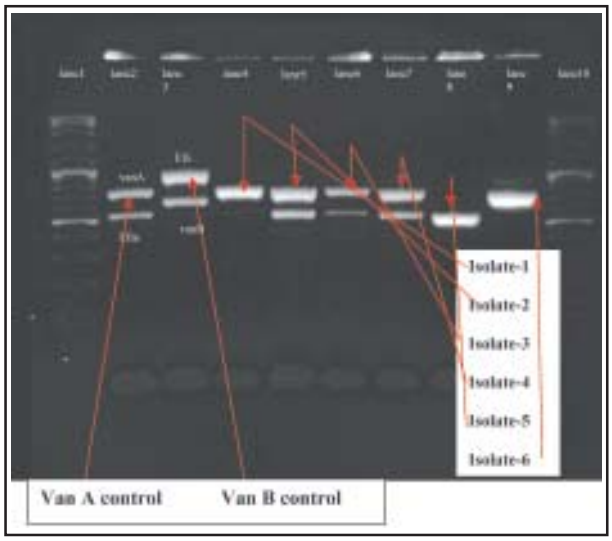

Fig-1: PCR reaction shows detection of *vanA* gene from six VRE isolated at HKL, Malaysia.

Table-I: Determination of Vancomycin resistance enterococci by disk diffusion.

Antibiotic	*Susceptible No. (%)*	*Resistant No. (%)*
Ampicillin	452(77%)	133(23%)
Gentamicin(120)	347(60%)	238(40%)
Vancomycin	579(99%)	6(1%)
Teicoplanin	579(99%)	6(1%)
Linezolid	585(100%)	0

ceptible to Linezolid.

In Table-II vancomycin resistance pattern is shown in different specimens. It reveals that out of 6 Vancomycin resistance enterococci 2 were from urine samples 3 from blood and 1 was from tissue samples.

Table-III describes diseases diagnosed in relation to the isolation of Vancomycin resistant bacteria and associated risks factors. Diseases and conditions identified were end stage renal failure (3) one with catheter related bloodstream infection, interstitial lung disease with cardiac complications and respiratory failure, diabetes mellitus, Parkinson disease with pneumonia, end-stage renal failure with catheter related bloodstream infection, end-stage renal failure and nephritic syndrome. Most detectable risks factors were catheter related bloodstream infections and prolonged intubation and prolonged hospitalization, recurrent hospitalization and dialysis, previous exposure to Vancomycin and Cephalosporin.

Molecular characteristics of vancomycin-resistant enterococci identified at HKL, Malaysia: Polymerase chain reaction was performed on 6 strains where the MIC of vancomycin > 32 μg/ml. From the results of agarose gel electrophoresis of PCR products it was revealed that all the VRE isolates were identified to carry Van A gene.

DISCUSSION

The acquisition of Vancomycin resistance by enterococci has seriously affected the treatment and infection control. In our study out of all isolates six (1%) were confirmed as vancomycin-resistant *enterococci*. These 6 VRE isolates were further identified as *E. faceium* (4/6), *E. faecalis* (1/6) and *E. avium* (1/6). Polymerase chain reaction for all these VRE isolates showed *vanA* gene.

VRE infections are more than double the risk of death compared with other infections, cause serious complications, and require longer hospital stay.[8] To our knowledge, this study is the first to define the prevalence of VRE at HKL, Malaysia where only 1% of the strains showed resistance to glycopeptides. This low prevalence of vancomycin resistance is also demonstrated in Turkey.[9] VRE prevalence was 9.5% at the center affiliated with the university of Maryland hospital 9% among 111 dialyses patients near New York City 6% at the Vanderbilt University Medical Center and 8.1% at Johns Hopkins University Hospital.[10] The alarming point about the spreading potential of resistance is that the Vancomycin resistance genes could be transferred among enterococci and from enterococci to staphylococcal species.[11]

In 2006, the European Antimicrobial Resistance Surveillance System (EARSS), a network of national surveillance systems, reported vancomycin-resistance rates among enterococci ranging from none in Iceland, Norway, Romania, Bulgaria, Denmark and Hungary, to 42% of *Enterococcus faecium* strains in Greece.[12] The prevalence of vancomycin resistance among enterococci significantly increases with the number hospital beds.[13] In our study, most of the *enteroccoccus* spp were isolated from patients with urinary tract infections (21%) followed by those with end stage of renal diseases (17%).

In the present study, most detectable risks factors were catheter related bloodstream infections and prolonged intubation and prolonged hospitalization, recurrent hospitalization and dialysis and previous exposure to Vancomycin and Cephalosporin.

Reports with respect to risk factor had shown that Vancomycin use was a major risk factor for the VRE infection or colonization.[14] An increased risk of VRE infection and colonization had been associated with reckless use of antibiotics and long hospitalization.

Table-II: Determination Vancomycin and other antibiotic resistance enterococci in different specimens.

Specimen	*Ampicillin*	*Gentamicin(120)*	*Vancomycin*	*Teicoplanin*
Urine	116	220	2	2
Blood	12	13	3	3
Pus	4	4	0	0
Tissue	1	1	1	1
Total (%)	133(23 %)	238(40 %)	6(1%)	6(1%)

Table-III: Clinical characteristics of vancomycin-resistant enterococci (VRE) infections in 6 confirmed cases at HKL, Malaysia.

Case	*Age*	*Specimen*	*ward*	*Diagnosis*	*Date of isolation*	*Risks factors*
1	70	Blood from catheter, peripheral negative	UrologyICU	End stage renal failure	1/11/07	* Previous vancomycin administration for CRBSI * Recurrent hospitalizations
2	55	Urine	ICU	Interstitial lung disease with cardiac complications and respiratory failure	12/11/07	* Prolonged intubate > 3 weeks, * Broad spectrum antibiotics >2 weeks * Prolonged hospitalization >1 month with urinary catheter
3	68	Tissue(wound)	GICU	D M, Parkinson with Pneumonia	11/02/08	* Previous vancomycin for MRSA, · * Prolonged hospitalization > 1 month
4	73	Blood from catheter, peripheral (CONS)	Urology	ESRF with Catheter related bloodstream infection (CRBSI)	21/02/08	* ESRF * Recurrent hospitalization for dialysis, * Previous exposure to Cephalosporin, vancomycin for CRBSI
5	49	Blood	UrologyICU	ESRF	29/03/08	* ESRF, · * Previously on Vancomycin * Recurrent hospitalization
6	44	Urine	Urology	Nephrotic syndrome	18/04/08	* Nephrotic syndrome with fluid overload, * Recurrent hospitalization

Legends: ESRF=end-stage renal failure, CRBSI=catheter related bloodstream infections, IV=intravenous, CONS=coagulase negative Staphylococcus.

In previous studies, VRE colonization was associated with prolonged length of hospital stay, previous admission to an intensive care unit (ICU), and severe underlying disease.[15] In addition; evidence showed that longer hospitalization and ICU stay are possible risk factors to get colonized with multiple clones of VRE.[16] Extensive or multiple hospitalizations show a correlation between the subsequent development of VRE infections, prior antibiotic treatment, prolonged stay within intensive care units and, in some instances, even intra-hospital transfers.[17] A study conducted from 2003 to 2004 showed that prior use of antimicrobial therapy, including Vancomycin and Cephalosporin, has been shown to be associated with acquisition of VRE.[18] Also, the presence of underlying disease was significantly associated with an increased risk for VRE colonization. Of note, impaired renal function and hemodialysis have been previously implicated as risk factors in VRE outbreaks. The use of vancomycin increased the risk of colonization 2.5-fold and third generation cephalosporins 2-fold.[14]

Other risk factors that have been described in this study like cephalosporin usage, use of carbapenem, malignancy, instrumentations like catheter and previous hospitalization were not statistically significant. Study on VRE bacteremia in oncology ward found that the use of antimicrobial agents with activity against anaerobes (metronidazoles, clindamycin and imipenem) determined a risk factor for the development of vancomycin-resistant bacteriaemia.[18]

Polymerase chain reaction was performed on 6 strains where the MIC of vancomycin > 32 µg/ml. All of these VRE, (100%) isolates were identified as having the *VanA* genotype (Fig.1). Sixty seven percent (4/6) of the VRE were identified as *E. faecium* and others were identified as *E. avium* and *E. faecalis*. *E. faecalis* is more common in human infections.

VanA is considered as the predominant type of resistance reported in Europe and is characterized by acquired inducible resistance to both vancomycin and teicoplanin and is transferable to other significant pathogens, like methicillin-resistant *Staphylococcus aureus*, due to its location on conjugative plasmids. In another report both in Europe and USA V*anA* was detected as the most common type of vancomycin resistance gene though a vast majority of VRE isolates in Singapore belongs to *vanB* gene.[19] Most Vancomycin-resistant *E. faecium* strains isolated in Korea showed the *Van A* phenotype, which was defined as having high level resistance to vancomycin and teicoplanin [11]

Between 1998 to 2004, the study of VRE was conducted in four tertiary hospitals in Korea and reported that 92 of 98 vancomycin-resistant enterococci isolates (93.9%) showed the *van A* phenotype with resistance to both vancomycin and teicoplanin, where six strains were resistant to vancomycin but susceptible to teicoplanin [16]

In our study, we found Vancomycin-resistant *E. avium* which was isolated from a blood culture of an elderly man with end stage renal failure, exhibited lower MIC of vancomycin (84µg/ml) compared to the other isolated VRE cases. This strain is usually isolated from animal or environmental sources, however we are not sure how this patient acquired this species.

Vancomycin-resistant enterococci are an underrecognized problem among patients in HKL, Malaysia, especially in renal units. After first detection VRE at HKL in the year 2006[5] no study was conducted on it in the hospital. In the present study, we found prevalence of 1% (6/585) prevalence of VRE. The VRE cases were mostly from patients with renal disease (50%) who had recurrent hospital admissions and were exposed to multiple antimicrobials including vancomycin. *Enterococcus faecium* was found to be the most common species among the isolated VRE cases that also exhibited multi-resistant antibiogram and showed high level MIC for vancomycin (> 256µg/ml) and all of them have *Van A* phenotypes. The present study would create awareness to the clinicians about VRE and their detrimental effect to the infected patients.

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