Comparative study between molecular and non-molecular methods used for detection of Vancomycin Resistant Enterococci in Tanta University Hospitals, Egypt

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Abstract: Introduction: Enterococci have become resistant to a wide range of antibiotics which include aminoglycosides and glycopeptides like vancomycin. The rapid increase of vancomycin resistance compromises physicians' ability to treat infections caused by these strains because the therapeutic options for VRE infections are very limited. **Methods:** The present study included 112 hospitalized patients having nosocomial infections. Selective culture was done on bile esculinazide agar for all suspicious colonies. Enterococcal species were identified using the VITEK-2 system. Antibiotic susceptibility pattern for enterococcal isolates was done using disc diffusion method. Chromogenic medium used for screening VRE, The MIC of vancomycin was determined by E test and PCR was done for detection of vanA gene. **Results:** Out of 112 patients, 32 enterococci species (28.6%) were isolated. Most commonly isolated species were *E. feacalis* (53%), followed by *E. facium* (40.6%), *E. avium*(3.1%), and *E. durans* (3.1%). VRE strains were *E. facium* (83.3%) and *E. feacalis* (16.7%). By disc diffusion method, 34.4% of isolated enterococci were VRE. The same percentage was detected by Chrome agar. Lower percentage (18.8%) was detected by Vitek2 and E-test. Van A gene could be detected in 18.8% of enterococci. The highest sensitivity and specificity (100%) was proved by both E-test and Vitek2 and specificity (92%), Chrome agar showed 100% sensitivity but 81% specificity. However, disc diffusion method showed 83.3% sensitivity and 77% specificity. Accuracies of VRE detection by disk diffusion method, chrome agar, E-test method, and Vitek 2 system were 78%, 84%, 100%, 100% respectively. **Conclusion:** PCR assay are in agreement with E-test and Vitek2 automated system employed for identification and test susceptibility of clinical *Enterococcus* spp. However, disk diffusion method proved to be less reliable for detection of resistance and should be replaced by routine MIC testing.
[Marwa Mostafa Shalaby, Kareman Ahmed Eshra, and Wageih Salem El-Naghy, Radwa Mahmoud El-Sharaby. **Comparative study between molecular and non-molecular methods used for detection of Vancomycin Resistant Enterococci in Tanta University Hospitals, Egypt.** *Life Sci J* 2016;13(1s):71-78]. ISSN 1097-8135 (print); ISSN 2372-613X (online) http://www.lifesciencesite.com. 8. doi:10.7537/marslsj1301s1608.

Keywords: Enterococci, Multidrug resistant enterococci, VRE, Chromogenic media, Infection control of VRE, vanA gene, PCR.

1. Introduction

Enterococci are part of normal intestinal flora of humans and animals, however they have emerged as community acquired pathogens and an important cause of health care associated infections ***(Jada and Jayakumar, 2012).*** A National Nosocomial Infections Surveillance revealed that *enterococci* remain in the top 3 most common pathogens that cause health care associated infections after *Escherichia coli* and *Staphylococcus aureus* ***(NNIS, 2010).***

Two most common species, *Enterococcus faecalis*and *Enterococcus faecium,* are among the leading causes of several human infections, including bacteremia, septicemia, endocarditis, urinary tract infections, wound infections, neonatal sepsis and meningitis ***(Hossein and Mohammad, 2014)***.

Many factors are responsible for the virulence of the organism ***(Jawad et al., 2010).*** A number of studies have identified different virulence factors including gelatinase, cytolysin, enterococcal surface protein (Esp), aggregation substance(AS), microbial surface component recognizing adhesive matrix molecule adhesin of collagen from enterococci (MSCRAMM Ace), pili, serine protease, cell wall polysaccharide and superoxide ***(Giridhara et al., 2009).***

Enterococci can survive for long time on environmental surfaces. They are resistant to heat, chlorine and some alcohol preparations which may explain why these organisms are widely disseminated in the hospital setting ***(Austin et al., 1999).*** Transmission of VRE can occur through direct contact with colonized or infected patients or through indirect contact via the hands of health-care workers (HCWs), or equipments or environmental surfaces ***(Song et al., 2008)***.

Enterococci display a variety of mechanisms for acquired and intrinsic resistance. They have remarkable genome plasticity and utilize plasmids, transposons, and insertion sequences to efficiently acquire and transfer mobile resistance elements, facilitating dissemination of resistance genes ***(Cattoir***

and Leclercq, 2013). *Enterococci* have become resistant to many antibiotics which include Beta lactam antibiotics, aminoglycosides, and most importantly glycopeptides like vancomycin***(Medeiros et al., 2014).*** The emergence of high-level aminoglycoside resistant (HLAR) *enterococci* and vancomycin-resistant *enterococci* (VRE) causes great difficulties in treatment. The risk also exists for VRE to transfer vancomycin resistance genes to other Gram positive organisms, including *staphylococci* ***(Wanxiang et al., 2015).***

Vancomycin should be used to treat infections with strains that exhibit high-level resistance to ampicillin. Vancomycin is indicated for the treatment of serious infections by Gram-positive bacteria which are unresponsive to other less toxic antibiotics. It is often reserved as the "drug of last resort", used only after treatment with other antibiotics had failed ***(Batchelor et al., 2010).***

Enterococci were the first pathogens that showed acquired vancomycin resistance. Resistant strains have been isolated from clinical samples in Europe and the USA in the late 1980s ***(Leclercq et al., 1989 and Sahm et al., 1989).*** There are two types of vancomycin resistance in *enterococci*. Intrinsic resistance is seen in *E. gallinarum*, *E. casseliflavus* and *E. flavescens*. This type demonstrates an inherent low-level resistance to vancomycin. However, Enterococcican also acquire resistance to vancomycin by acquisition of genetic information from another organism. This resistance is most commonly seen in *E. faecium*and *E. faecalis*, but also has been recognized in *E. raffinosus*, *E. avium*, *E. durans*, and several other enterococcal species ***(Paulsen et al., 2003).***

Eight phenotypic variants of acquired glycopeptide resistance in *enterococci* have been described (Van A, Van B, Van D, Van E, Van G, Van L, Van M, and Van N), with one type of intrinsic resistance (Van C) being unique to *E. gallinarum* and *E. casseliflavus* ***(Xu et al., 2010).*** A change in the precursor to DAla-D-Lac (Van A, Van B, Van D, VanM) causes a 1,000-fold decrease in affinity for vancomycin, and a change to D-Ala-D-Ser (VanC, Van E, Van G, Van L, Van N) causes a 7-fold decrease in affinity for vancomycin. Van A is responsible for most of the human cases of VRE around the world, and is mostly carried by *E. faecium* ***(Fisher and Phillips, 2009).***

Some strains of Van A and Van B-type VRE *enterococci* are not just resistant to vancomycin but also require it for growth. This phenomenon is called vancomycin dependence. These *enterococci* mayturn-off their normal production of D-Ala- D-Ala and then can grow only if a substitute dipeptide like structure is made (***Kerbauy et al., 2011***).

Enterococcal infections can be diagnosed by either phenotypic methods, which are time consuming, or molecular methods requiring costly equipment and highly trained staff. An accurate, rapid diagnostic test has the ability to greatly reduce the spread of this organism which is capable of colonizing patients for long periods, potentially even lifelong ***(Griffith et al., 2013).***

Many automated commercial systems (such as the Vitek system and the MicroScan rapid system) have evaluated. It has been shown that these methods are not reliable for the detection of vancomycin resistance in *enterococci* especially low level vancomycin resistance ***(Okabe et al., 2002).***

PCR protocols to identify VRE from stool samples have been evaluated ***(Kariyama et al., 2000).*** These are very helpful for surveillance of VRE using rectal swabs and stool samples and are less time consuming. Once standardized, these are also less expensive than the traditional culture screening methods. Multiplex PCR can be useful to detect which of the van genotypes is present in a particular isolate ***(Sujatha and Praharaj, 2012).***

The treatment of VRE infections is based on infection severity and *in vitro* susceptibility to other antibiotics. For example, uncomplicated urinary tract infections have been treated successfully with nitrofurantoin. Isolates that remain susceptible to penicillin or ampicillin (MICs of 0.5-2 mcg/mL) may be treated with high doses of these agents. Doxycycline, chloramphenicol, and rifampin in various combinations have been used to treat VRE infections, but the newer antibiotic choices as Quinupristin/dalfopristin, Oxazolidinones, Daptomycin, Tigecycline, Lipoglycopeptides, Telavancin, dalbavancin, and Oritavancin are also now available ***(Singh et al., 2007).***

2. Methods

Patients

The present study included 112 hospitalized patients having nosocomial infections (infections that became evident 48 hours or more after hospital admission and that was not present or incubated at time of admission). Their ages ranged from 5 years to 62 years.

Specimens collection, processing and identification of enterococci

After collecting demographic data and history records of patients, urine, endotracheal aspirates, wound swabs, blood, and other specimens were taken aseptically according to the type of infection. Identification of *enterococci* to the Genus Level by colonial morphology, Microscopic examination, and biochemical tests e.g Catalase negative. Identification was confirmed by Growth in 6.5% NaCl, Ability to

grow at 10°C and 45°C, Ability to hydrolyze esculin in the presence of bile using selective bile esculinazide agar. Identification of enterococci to the species level using the VITEK-2 COMPACT system using GP cards (Biomerieux).

Detection of antibiotic susceptibility pattern for enterococcal isolates:

Antibiotic susceptibility testing for different antibiotics was done using disc diffusion method according to the Kirby-Bauer disc diffusion method using Penicillin (10unit), Ampicillin (10µg), Vancomycin (30µg), Teicoplanin (30µg), Erythromycin (15µg), Tetracycline (30µg), Rifampicin (5µg), Chloramphenicol (30µg), Ciprofloxacin(5 µg), Linezolid (30 µg), levofloxacin (5 µg), quinupristin-dalfopristin (15 µg), Gentamicin 120 µg, and Streptomycin 300 µg discs (oxoid)..

Detection of Vancomycin resistance:

Chromogenic medium (Chromatic™ VRE) (bioMèrieux, France) was used for screening vancomycin resistant *enterococci.* This medium is aSelective chromogenic medium for the detection and differentiation of *Enterococcus faecium* and *E. faecalis* showing acquired vancomycin resistance. The Minimum Inhibitory Concentration (MIC) of vancomycin was determined by Epsilometer (E) test (LIOFILCHEM® - ITALY) and by VITEK-2 COMPACT system. Detection of vancomycin resistance gene (Van A) was done……

Detection of vanA gene by PCR

Enterococcus spp. genomic DNA was extracted by the MagNA pure compact nucleic acid isolation kit I in combination with MagNA pure Bacteria Lysis Buffer and the DNA bacteria purification protocol (Clinilab). PCR assay was carried out using species-specific primer: vanA gene primer with Nucleotide sequence (5'- 3') a (GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA). The amplicon (bp) was 231 for E. faecium and 330 for E. faecalis (**Bell *et al.,* 1998**). All PCR amplifications were performed in a final volume of 20 µL containing one ρmol of the primer (Forward and Reverse), 0.17 mMdNTPs, 2.5 mM $MgCl_2$, one U of Taq DNA polymerase, buffer of Taq, and 10 µL template DNA. An initial cycle of denaturation (94 °C for two min), was followed by 30 cycles of denaturation (94 °C for one min), annealing at an appropriate temperature for one min and elongation (72 °C for 10 min). A Thermal Cycler (Biometra T personal thermocycler) was used to carry out the PCR reactions. PCR products were analyzed by gel electrophoresis in 1.5% agarose stained with ethidium bromide (0.5 g.mL-1), observed under UV transillumination (Illuminator UVstar 312nm Biometra) and photographed (canon).

3. Results

Out of 112 patients (61 males and 51 females) having nosocomial infections aged from 5 years to 62 years from different departments and ICUs, 32 enterococci species (28.6%) were isolated. Most commonly isolated species were E.feacalis (53%), followed by *E.facium* (40.6%), *E.avium* (3.1%), and *E. durans* (3.1%). VRE strains were *E.facium* (83.3%) and *E.feacalis* (16.7%) **(Table 3)** and were mostly isolated from urine, wounds, and endotracheal aspirates **(Table 2)**. Demographic data and risk factors for VRE infections among studied population are demonstrated in **table 1**. Most of patients with VRE infections were from ICUs with prolonged hospital stay (>7d). For detection of VRE strains, all enterococcal isolates were tested for vancomycin susceptibility by disk diffusion method, chrome agar for VRE, determination of minimum inhibitory concentration (MIC) by E-test method and Vitek 2system, and confirmed by detection of Van A gene by PCR. By disc diffusion method, 34.4% of isolated enterococci were VRE. The same percentage was detected by Chrome agar. Lower percentage (18.8%)was detected by Vitek2 and E-test. Van A gene could be detected in 18.8% of enterococci **(Table 4)**. Taking the PCR as a reference method, the highest sensitivity and specificity (100%) was proved by both E-test and Vitek2 and specificity (92%), Chrome agar showed 100% sensitivity but 81% specificity. However, disc diffusion method showed 83.3% sensitivity and 77% specificity. Accuracies of VRE detection by disk diffusion method, chrome agar, E-test method, and Vitek 2 system were 78%, 84%, 100%, 100% respectively**(Table 5).**

The results obtained in the study are summarized in the following tables:

4. Discussion

Enterococci are a main cause of nosocomial infections, especially urinary tract, the bloodstream and surgical sites infections ***(Calderon-Jaimes et al., 2003* and *Olawale et al., 2011)***. This may be due to their broad natural and acquired resistance to antimicrobial agents, including glycopeptides (vancomycin and teicoplanin). They have many antibiotic resistant genes. Hospital acquired VRE infections is a major problem as therapeutic options are very limited ***(Cetinkaya et al., 2000; Willems et al., 2005)***.

Table 1: Demographic data and risk factors for VRE infections among studied population

Parameter	VRE (n=6)		X^2	*p*
	No.	**%**		
Age				
5 – 25	1	16.7		
26 – 50	2	33.3		
>50	3	50.0		
Sex				
Male	3	50.0	0.046	1.000
Female	3	50.0		
Antibiotic administration				
No	2	33.3	0.011	1.000
Yes	4	66.7		
Duration of hospital stay				
≤7	1	16.7	1.510	0.399
>7	5	83.3		
Department of hospitalization				
ICU	5	83.3	6.285*	0.021*
Other hospital wards	1	16.7		
Intervention				
InvasiveProcedures	4	66.7	3.662	0.076
Noninvasive Procedures	2	33.3		
Associated disease				
Immunocompromized	3	50.0	0.046	1.000
Immunocompetent	3	50.0		

X^2: Chi square testMC: Monte Carlo forChi square test FE: Fisher Exact *: Statistically significant at $p \leq 0.05$

Table 2: Distribution of enterococci and VRE according to type of the specimen

	Patients (n=112)		Enterococcus isolates (n=32)		X^2	p	VRE (n=6)		X^2	*p*
	No.	**%**	**No.**	**%**			**No.**	**%**		
Urine	39	34.8	13	40.6			3	50.0		
Blood	19	17.0	6	18.8			0	0.0		
Wound	39	34.8	9	28.1	0.628	0.900	2	33.3	4.027	0.204
Endotracheal aspirate	15	13.4	4	12.5			1	16.7		

Table (3): Distribution of different Enterococcus species among VSE and VRE

***Enterococci* (n=**	N	VSE (n=22)		VRE (n=6)		VIE (n=4)	
		No.	**%**	**No.**	**%**	**No.**	**%**
E.Faecium	13	6	27.4	5	83.3	2	50.0
E.Faecalis	17	14	63.6	1	16.7	2	50.0
E.Avium	1	1	4.5	0	0.0	0	0.0
E.Durans	1	1	4.5	0	0.0	0	0.0
X^2(P)				**7.925(0.276)**			

Table 4: Comparison of VRE detection by different methods

Enterococci	**Disk diffusion**		**Chrome Agar**		**E- test for vancomycin**		**Vitek2 method**		**PCR**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Vancomycin resistant	11	34.4	11	34.4	6	18.8	6	18.8	6	18.8
Vancomycin susceptible	12	37.5	21	65.6	12	37.5	13	40.6	26	81.2
Vancomycin intermediate	9	28.1			14	43.7	13	40.6		
Total	**32**	**100.0**	**32**	**100.0**	**32**	**100.0**	**32**	**100.0**	**32**	**100.0**

N.B: intermediate resistant strains can't be detected by neither chrome agar nor PCR

Table 5: Sensitivity and specificity of chrome agar, disk diffusion, E- test, Vitek2 methods compared with PCR as a reference method

	Sensitivity	Specificity	PPV	NPV	Accuracy
Disk For Vancomycin	83.3	77	45	95	78
Chrome Agar For VRE	100	81	55	100	84
E- Test For Vancomycin	100	100	100	100	100
Vitek 2	100	100	100	100	100

Photo 1: Chrome agar positive growth of *E. facium* (violet) and *E. feacalis* (blue)

In this study, *enterococci* isolates were 32 (28.6%) from total 112 Samples. Similar results were obtained by ***Ashour et al. (2004)*** who found that *enterococci* represented 27.4% among total specimens examined in their study. VRE were mostly from urine samples (50.0%), followed by wound and endotracheal aspirate (33.3%, 16.7%). ***Young et al. (2009)*** also showed that 92%of VRE in their study were isolated from urine specimens, and the remaining 8% were isolated from blood, stool, and cerebrospinal fluid, respectively.

Using VITEK2 compact system, our study revealed that the most common isolated Enterococcus species was *E. faecalis* (53.1%) followed by *E. faecium* (40.6%), *E. durans*(3.1%), *E. avium* (3.1%). Among VRE, *E. faecium* had the highest rate (83.3%) of all VRE isolates followed by *E. faecalis*(16.7%). Higher rates of *E. faecium* among VRE isolates also were reported by other studies. In Czech Republic, ***Kollef et al. (2006)*** found that *E. faecium* was the most common isolate (78%) among VRE isolates. Another study performed by ***Littvik et al. (2006)*** in Cordoba City, Argentina reported that *E. faecium* accounted for 94.4% of vancomycin VRE. Contrarily, Getachew ***and his collegues. (2012)*** reported that VRE species isolated were *E. faecalis* (48%), followed by *E. faecium* (25.7%), *E. gallinarum* (12.1%) and *E. casseliflavus* (1.4%).

On studying the relation between demographic data and isolation of VRE, the present work showed that the 50% of VRE was found in patients with age group above 50 years. ***Salem-Bekhit et al. (2012)***, also found that patients with VRE were above 56 years. Patients with prolonged hospital stay are more susceptible to cross transmission of pathogens and to a greater use of antimicrobials with selective pressure. Our study revealed that although non statically significant, higher rates of VRE were correlated with prolonged hospital stay and antibiotic administration as 66.7% of VRE patients were taking antibiotic and 83.3% of VRE patients were hospitalized more than 7 days. In accordance with these results, ***Ibrahim et al. (2011)*** also found that the highest rate of isolation of *enterococci* was observed in patients with long duration of hospital stay being 61% in patients with duration ≥ 10 days, 19% in patients with duration 6 - 10 days and only 15% if the duration ≤ 6 days. We also revealed that VRE infections were associated with admission to an intensive care unit (ICU) 83.3% of VRE patients were hospitalized in (ICU) with statistically significant difference.

Of the 32 enterococcal isolates obtained in our study, 84.4, 100, 90.6, 87.5 and 37.5 % were resistant

to penicillin G, ampicillin, erythromycin, tetracycline and chloramphenicol respectively. A similar resistance rate to ampicillin was reported by ***Mohammad et al. (2008)*** (100%). but lower results (66%) were reported by ***Mohanty et al. (2005)*** in a study performed in tertiary care hospital in India. In the current study, ciprofloxacin and Levofloxacin resistance was 75%. A similar rate was reported by ***Stefani et al. (2012)*** (72%).

For detection of VRE strains, all enterococcal isolates were tested for vancomycin susceptibility by disk diffusion method, chrome agar for VRE, determination of minimum inhibitory concentration (MIC) by E-test method and Vitek 2 system, and confirmed by detection of Van A gene by PCR. By disc diffusion method, 34.4% of isolated enterococci were VRE. The same percentage was detected by Chrome agar. Lower percentage (18.8%) was detected by Vitek2 and E-test. Van A gene could be detected in 18.8% of enterococci. Taking the PCR as a reference method, the highest sensitivity and specificity (100%) was proved by both E-test and Vitek2 and specificity (92%), Chrome agar showed 100% sensitivity but 81% specificity. However, disc diffusion method showed 83.3% sensitivity and 77% specificity. Accuracies of VRE detection by disk diffusion method, chrome agar, E-test method, and Vitek 2 system were 78%, 84%, 100%, 100% respectively**.**

There are some errors associated with disk diffusion susceptibility testing against vancomycin. Therefore, to depend only on report of disk diffusion test against vancomycin may result in unnecessary elimination of the antibiotic as a part of treatment schedule ***(Peter et al., 2013)***. Therefore, a routine MIC monitoring of important antibiotics like vancomycinis recommended, before reporting it as resistant or intermediately sensitive ***(Sreeja et al., 2012)***.

In the current study, VRE chrome agar (bioMèrieux) demonstrated (84%) accuracy,(100%) sensitivity and (81%) specificity in detection of resistance. Also, ***kuch et al. (2009)*** found that the specificity varied between (60- 99.4%). In accordance to ours, ***Kumar et al. (2008)*** findings was that chromogenic media sensitivity was (96.9%) but he reported (99.4%) specificity. The obtained results in this study indicated that CHROM agar method is an easy to use, cost-and time- effective procedure especially in admitted patients where the routine screening may be required.

Gülmez and Hasçelik (2011) compared the Phoenix system and microdilution method and observed an excellent agreement for all of the antibiotics with category agreement rates of>97%. We reported similar results of Vitek2 and E-test which show excellent agreement with PCR results indicating that they are reliable methods. In our study we detected Van A gene in 6 strains of enterococci. Also, ***Luciana et al., 2014***, revealed that the *vanA* gene was predominant in *E. faecium* tests since this gene was detected in 100% of vancomycin-resistant isolates in their study.

The primary objective of the study was to compare between different molecular and non-molecular methods used for detection of VRE. This study revealed that the PCR assay are in agreement with E-test and Vitek2 automated system employed for identification and test susceptibility of clinical *Enterococcus* spp. However, disk diffusion method proved to be less reliable for detection of resistance and should be replaced by routine MIC testing.

Conclusions

Emergence of multi-drug resistant *enterococci* in this study, particularly to high level aminoglycoside and vancomycin is an alarming situation that raises the concern about the use of glycopeptides as an appropriate choice for enterococcal infections. We recommend that enterococcal isolates should be regularly screened for vancomycin and high level aminoglycoside resistance. All VRE isolated in this study were susceptible to linezolid indicating it as an appropriate therapeutic option.

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1/25/2016