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Full Length Research Paper

Molecular characterization of enterococcus strains isolated from cases of neonatal sepsis in neonatal intensive care unit

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This work aimed at determining the occurrence, species prevalence, antibacterial resistance, and genetic characteristics of vancomycin-resistant enterococci (VRE) isolated from cases of neonatal sepsis at neonatal intensive care unit (NICU). Out of 134 blood samples of neonatal sepsis enterococci were isolated from 111 cases. Of these 111 strains VRE strains were identified and the resistance genotype was determined by polymerase chain reaction (PCR). The results of the study showed that 3 isolates (2.98%) were identified as VRE in cases of early onset sepsis and 5 isolates (4.47%) were identified as VRE in cases of late onset sepsis. The majority of the isolates were resistant to erythromycin (57.7%), ciprofloxacin (45.9%), where all isolates were susceptible to linezolid except one isolate (0.9%) of *Enterococcus faecium*. This study showed that 4 strains of *Enterococcus faecalis*, and all of *E. faecium* and *Enterococcus gallinarum* strains were positive for VanA genotype. VanB products were detected in three isolates (two strains of *E. feacalis* and one strain of *E. feacium*), and PCR of the VanC gene was obtained from two isolates (one *E. gallinarum* specimen, and one *E. feacium* specimen). This study showed an emergence of VRE along with increased rate of multidrug-resistant enterococci in cases of neonatal sepsis. Regular surveillance of antimicrobial susceptibilities should be done regularly and the risk factors should be determined.

Key words: Vancomycin-resistant enterococci (VRE), vancomycin, neonatal sepsis, neonatal intensive care unit (NICU).

INTRODUCTION

Neonatal sepsis is a clinical syndrome characterized by systemic signs of infection accompanied by bacteremia in the 1st month of life. According to National Neonatal Perinatal Data (NNPD 2002-03) incidence of neonatal sepsis is 30/1000 live birth National Neonatology Forum of India (2004). Neonatal sepsis is classified as either early or late based on the timing of presentation, with early onset sepsis ranging from 48 h to 6 days after delivery and late onset sepsis generally occurs beyond the first week of life (Shashikala et al., 2002).

The microbial etiology of neonatal sepsis is variable and often changes temporarily. Group B *streptococci* is a common cause of neonatal sepsis in west but infrequent in tropical countries. Commonly, *Staphylococcal aureus*, *klebsiella*, *Escherichia coli* along with *coagulase negative staphylococcus* and *pseudomonas* are the main organisms responsible for neonatal septicemia worldwide (Rao et al., 1993). In view of the fulminating course of neonatal septicemia every attempt should be made for early diagnosis and management (Sinha et al., 1986).

Enterococci have evolved over the past century from being an intestinal commensal organism of little clinical significance to becoming the second most common nosocomial pathogen associated with significant morbidity and mortality (Giridhara et al., 2009). In recent years, there has been a rapid increase in the incidence of neonatal septicemia caused by vancomycin-resistant enterococci (VRE). The resistance may be intrinsic or acquired via gene transfer (Tallur et al., 2009).

Widespread use of vancomycin and extended-spectrum

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Table 1. Nucleotide sequences of PCR primers used for amplification of vancomycin-resistant enterococci genes.

Primers pairs	Primer sequence (5´-3´)	Product Size (bp)
A1	5´-GGGAAAACGACAATTGC-3´	732
A2	5´-GTACAATGCGGCCGTTA-3´	
B1	5´-ATGGGAAGCCGATAGTC-3´	635
B2	5´- GATTTCGTTCCTCGACC-3´	
C1	5´-CTCCTACGATTCTCTTG -3´	822
C2	5´- CGAGCAAGACCTTTAAG -3´	

(Dutka-Malen et al., 1995).

cephalosporins in hospitals likely contributed to the emergence and dramatic increase of VRE over the past 20 years (Donabedian et al., 2010).

Although more than one dozen species of enterococci have been identified, *Enterococcus faecalis* was the most common species associated with neonatal septicemia, followed by *Enterococcus faecium*. Infections caused by VRE were found to be associated with adverse outcome such as extended length of hospital stay, increased cost and increased mortality (Udo et al., 2003).

The glycopeptide vancomycin is the first choice alternative to penicillin-aminoglycoside combination for treatment of systemic enterococcal infections. Different types of vancomycin resistance genes have been reported in enterococci. Glycopeptide-resistant genotypes in enterococci include VanA (high-level resistance), which is detected in a wide variety of enterococcal species, VanB, VanB2 and VanD with moderate to high-level resistance and VanC (C1, C2, C3) causing intrinsic low-level resistance (Satake et al., 1997). Vancomycin resistance is most commonly found in *E. faecium* and is encoded by the *vanA* gene cluster carried on the mobile genetic element Tn1546 (Pantell et al., 2004). Transfer of resistance can occur via conjugative plasmids. Monitoring the antibiotic resistance of enterococci isolated from clinical specimens is a useful tool to get information about the prevalence of VRE and will be essential for controlling the spread of bacterial resistance. Despite the increasing reports of VRE in different countries, there is a distinct lack of data regarding the molecular characterisation of VRE isolates. The aim of this study was to determine the occurrence, species prevalence, antibacterial resistance, and genetic characteristics of VRE strains isolated from cases of neonatal septicemia in Tanta University Hospital

MATERIALS AND METHODS

Collection of specimens

134 cases of neonatal sepsis; 75 cases of early onset sepsis and 59 cases of late onset sepsis were included in the over a period of one year extended from March, 2011 to March, 2012. All cases were admitted to neonatal intensive care unit (NICU) in Tanta University Hospital. This work was done with approval of Research Committee in Faculty of medicine, Tanta University. 5 ml bloods were collected from each case at the day of admission. Identification of enterococci was based on their growth characteristics on blood agar, Gram staining, the catalase reaction, ability to grow in 6.5% NaCl broth and bile esculin hydrolysis .After the identity of the isolates was confirmed, they were stored in trypticase soy broth containing 16% glycerol at −70°C in freezer vials pending for further analysis.

Antimicrobial susceptibility testing

The susceptibilities of all isolates of enterococci to different antimicrobial agents were tested by the disc-diffusion method as standardized by the Clinical Laboratory Standards Institution (CLSI) Clinical Laboratory Standards Institution (2004). The following antimicrobial discs and concentrations were used: ampicillin (10 µg), vancomycin (30 µg), teicoplanin (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), amikacin (200 µg), gentamicin (10 µg), kanamycin (200 µg), and linezolid (30 µg) (Becton Dickinson Microbiology Systems, BBL, Cockeysville, MD, USA). The specific vancomycin-resistant genotype *(vanA*, *vanB* or *vanC)* was determined with polymerase chain reaction (PCR) analysis by using specific primers selected from published gene sequences.

DNA isolation and detection of vancomycin- resistance determinants

Isolates of VRE were cultured in 30 ml of brain heart infusion broth (Difco Laboratories). DNA was isolated as described previously by (Crosa and Falkow, 1981). Genes encoding the vancomycin resistance determinants, *vanA*, *vanB*, *and vanC* , were investigated by PCR using specific primers, as described by (Dutka-Malen et al., 1995) (Table 1). Amplification was performed using a kit from Gibco-BRL. A Perkin Elmer 9600 thermocycler was programmed for 32 cycles with the following parameters: denaturation at 94°C for 3 min, annealing at 60°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 2 min. Amplicons were analysed by electrophoresis on 1% agarose gels (Hispanagar; Sphaero Q) containing ethidium bromide in tris-acetate- ethylenediaminetetraacetic acid (TAEDTA) buffer for 2 h at 70 V in the presence of a 100-bp DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands).

RESULTS

This study was carried out on 134 case of neonatal septicemia attending neonatology unit in Pediatric Department of Tanta University Hospital, patients in this study were

Table 2. Distribution of patients as regard clinical diagnosis, gestational age, sex, body weight, and delivery mode.

Parameter	Early onset N = 75	Late onset N = 59
Gestational age Mean ±SD	37.52 ± 1.5	37.75 ± 1.29
Weight Mean ±SD	2.97 ± 0.29	2.98 ± 0.23
Sex		
Male	45	46
Female	30	13
Mode of delivery		
Vaginal	43	40
Casseran section	32	19

Table 3. Distribution of *Enterococcus* spp. in various clinical specimens.

Onset	No. of specimen	No (%) of *enterococcus* species			Total n (%)	No. of specimens with VRE n (%)
		***E. faecalis,* n (%)**	***E. faecium* n (%)**	***E. gallinarum* n (%)**		
Early onset	75	37 (49.33)	22 (29.33)	2 (2.66)	61 (45.52)	3 (2.98)
Late onset	59	33 (55.93)	13 (22.03)	4 (6.77)	50 (37.31)	5 (4.47)
Total	134	70 (52.23)	35 (26.11)	6 (4.47)	111 (82.83)	8 (7.46)

divided into two groups; group I formed of 75 cases complaining of early onset sepsis and group II formed of 59 cases complaining from late onset sepsis. Table 2 shows distribution of cases in the study as regard clinical diagnosis, gestational age, sex, body weight, and delivery mode. Table 3 shows distribution of *Enterococcus* spe-cies in the studied groups as it was found that in cases of early onset sepsis (group I) enterococci were isolated from 61 (45.22%) blood samples (45.52%), was 37 isolates (49.33%) *E. faecalis*, 22 isolates (29.33%) *E. faecium*, and two isolates (2.66%) *Enterococcus galli-narum.* out of these 61 isolates 3 isolates (2.98%) was identified as VRE. As regard group II, it was found that in cases of late onset sepsis enterococci were isolated from 50 (37.31%) blood samples 33 isolates (55.93%) *E. faecalis*, 13 isolates (22.03%) *E. faecium* and four isolates (6.77%) *E. gallinarum*. out of these 50 isolates 5 isolates (4.47%) was identified as VRE. The distribution of anti-microbial susceptibility patterns of isolated enterococci is summarized in (Table 4), The results show that the majo-rity of isolates were resistant to erythromycin (57.7%), ciprofloxacin (45.9%) and kanamycin (45.04%).

In addition, 35 (31.5%) of the isolates were resistant to ampicillin. The isolates were tested for their susceptibility to linezolid, a new oxazolidinone antibacterial. They were susceptible to linezolid except one isolate (0.9%) of *E. faecium*. This study investigated the prevalence of genes encoding vancomycin resistance in *enterococci* isolated from cases of neonatal septicemia.

The results showed that *van A* gene was found in 4 isolates of *E.feacalis*, two isolates of *E.faecium*, and one isolate of *E.gallinarum* , Van B was found in two the isolates of *E.feacalis*, one isolate of *E.faecium*, and was not found in any of the strains *of E.gallinarum* , and Van C was found in one strain of *E.faecium*, and one strain of of *E.gallinarum*, but not found in *E,feacalis* isolates (Table 5) (Figures 1, 2 and 3).

DISCUSSION

Out of 134 cases of neonatal sepsis included in the study *enterococcus* strains were isolated from 111 cases. Of these 111 strains of enterococci the vast majority of the isolates were either *E. faecalis* which caused about 52.23% infection or *E. faecium* which was responsible for about 26.11% of infection, while *E. gallinarium* accounted for only 4.47% of the isolates. The results of this study came in agreement with study performed by Mohanty et al, (2005) that found *E. faecium in* (22.9%) and *E. gallinarum* in (1.3%) of the isolates but was somewhat different as regard *E. faecalis* which was detected in (72.5%) of the isolates. Newborns most probably acquire these infections, from the vaginal and fecal flora of the mother and the environment where the delivery occurs that may explain this difference in isolated organisms (Mohanty et al., 2005). In reverse to this study Gheibi et al., (2008) found that *enterococcus* species were isolated from only 22% of cases of neonatal septicaemia,

Table 4. Resistance rates for clinical enterococcus isolates.

Antibiotic	MIC (µg/ml)	Resistant isolate			Total N = 111, n (%)
		E. faecalis N = 70, n (%)	***E. faecium*** N = 35, n (%)	***E. gallinarum*** N = 6, n (%)	
Ampicillin	> 16	11 (15.7)	22(62.9)	2 (33.3)	35(31.5)
Vancomycin	> 32	5 (8.6	2(8.6)	1 (16.7)	8(9.0)
Teicoplanin	> 32	4 (8.6	2(8.6)	1 (16.7)	7(9.0)
Erythromycin	ND	43 (60.8)	20(57.1)	1 (16.7)	64(57.7)
Ciprofloxacin	ND	35 (49.4)	13(37.1)	3 (50)	51(45.9)
chloramphenicol	ND	25 (35.5)	7 (20)	3 (50)	35(31.5)
Amikacin	> 512	15 (21.7)	9 (25.7)	2 (33.3)	26(23.4)
Gentamycin	> 500	16 (22.3)	4 (11.4)	1(16.7)	21(18.9)
Kanamycin	> 1000	44 (62.7)	5 (14.2)	1(16.7)	50 (45.04)
Linezolid	> 4	-	1 (2.85)	-	1(0.9)

Table 5. Distribution of Van producing *Enterococcus* isolates.

Isolate	Number of resistant strains (VRE)	Genotype		
		Genotype	NO	(%)
E. faecalis	5	Van A	4	80
		Van B	2	25
		Van C	-	0.00
E. faecium	2	Van A	2	100
		Van B	1	12.5
		Van C	1	12.5
E. gallinarum	1	Van A	1	100
		Van B	-	0.00
		Van C	1	12.5

Of the 111 isolates 57.7, 45.9 and 45.04% were resistant to erythromycin, ciprofloxacin and kanamycin, respectively, which are similar to the levels reported for these antibacterials among the enterococci isolated in Jordan by a study performed by Mahafzah et al. (2008). In this study, 57.7% of isolates were resistant to erythromycin, which is the same results reported in the study of Zouain et al. (2001) that found that 57% of the isolates were resistant to erythromycin .These results indicate diverse geographical distribution of erythromycin-resistant enterococci. The percentage of reported hospital *enterococcal* infections resistant to vancomycin increased from 0.3% in 1989 to 11% in 1996 (Karlowsky et al., 1999). In this study, VRE were found in 8 (7.46%) infants with neonatal septicemia and all were identified as *E. faecalis*, *E. faecium*, and *E.gallinarum*. 3 of these patients were complaining from early onset neonatal sepsis, and 5 cases were suffering from late onset neonatal sepsis. These results are in reverse with those of Udo et al. (2003) who detected VRE in 11 out of 415 isolates (2.6%) at Kuwait hospitals (8). Moreover, our results are also not in agreement with those of Jain et al. (2010) who did not report VRE in their results (Jain et al., 2010).

The results of this study showed that all of the isolates were susceptible to linezolid except one. Also, 90% of enterococcus isolate including *E. faecalis*, *E. faecium*, and *E. gallinarum* were vancomycin susceptible. The resistance rate to ampicillin was found to be 31.5% in enterococcal isolates. However, resistance rate to ampicillin reported by Mohanty et al. (2005) in a study performed in tertiary care hospital in India (66%) is higher than our result (Mohanty et al., 2005). This study investigated the prevalence of genes encoding vancomycin resistance in enterococci isolated from cases of neonatal septicemia. The results showed that *vanA* was found in 4 isolates of *E. feacalis*, two isolates of *E. faecium*, and one isolate of *E. gallinarum*, Van B was found in two of the isolates of *E. feacalis*, one isolate of *E. faecium*, and was not found in any of the strains *of E. gallinarum* and Van C was found in one strain of *E. faecium*, and one strain of *E. gallinarum*, but not found in *E feacalis* isolates.

In a study done by Arthur et al. (1993) seven *E. faecalis*

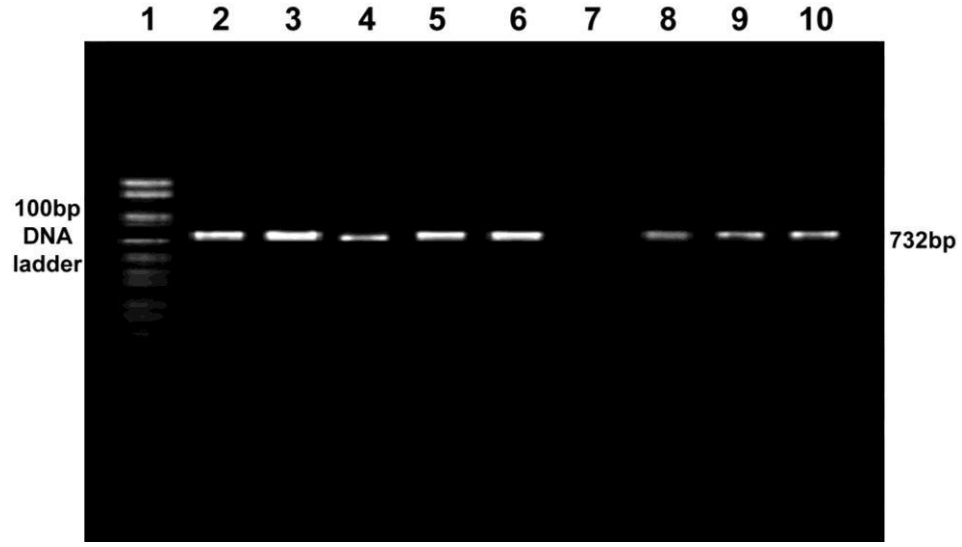

Figure 1. Agarose gel electrophoresis showing positive amplification of 732 base fragments specific for vanA of VRE . Lane 1, size marker (100-bp DNA ladder); lane 2, *E. faecium* ATCC 15559 (positive control); lanes 3 to 7, *E. faecalis* VRE; lane 8 and 9, *E. faecium* VRE and lane 10 *E. gallinarum.* (Van A present in 4 strains of *E. faecalis* , and 2 strains of *E. faecium* and one strain of *E. gallinarum.* The figure showed that 4 strains of *E. faecalis*, and all of *E. faecium* and *E. gallinarum* strains were positive for VanA genotype (732-bp) PCR product.

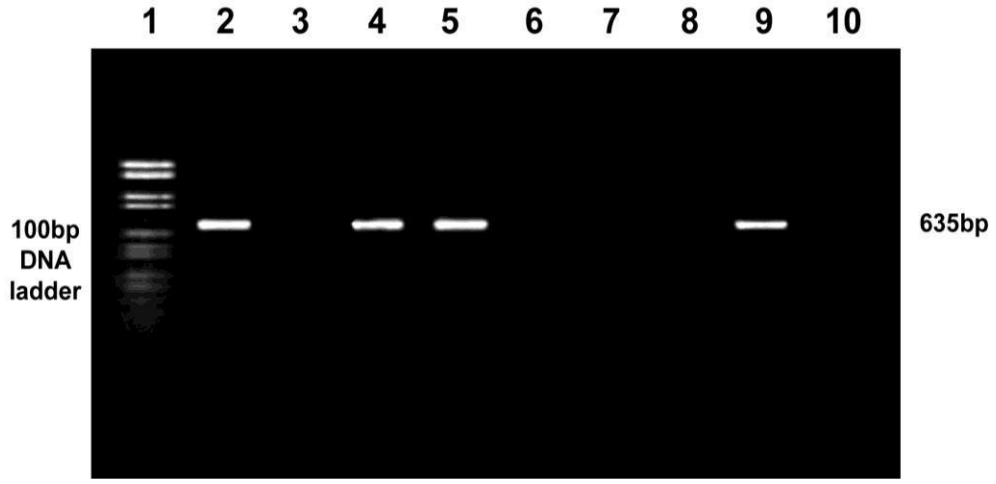

Figure 2. Agarose gel electrophoresis showing positive amplification of 635 base fragments specific for vanB of VRE from clinical specimens. Lane 1, size marker (100-bp DNA ladder); lane 2, *E. faecium* ATCC 29212 (positive control); lanes 3 to 7, *E. faecalis* VRE; lane 8 and 9, *E. faecium* VRE and lane 10 *E. gallinarum.* the figure showed VanB products (635 bp) were detected in three isolates (two strains of *E. feacalis* and one strain of *E. feacium*).

and one *E. faecium* isolates expressed vancomycin-resistance patterns compatible with the VanA and VanB phenotypes, respectively, and all gave positive results in PCR experiments for the *vanA* and *vanB* genotypes (Arthur et al., 1993), the results which came in accordance to our results to great extent. However, different studies showed variable results concerning the detection rates of VanA and VanB. In another study done by udo et al. (2003), that came in reverse to the results of our study, as they found that all VRE strains carried the VanA genotype, non VanB product was detected in any of the isolates. In contrast to Nelson et al. (2000) who reported that the majority of isolates (97%) were VanB positive and the remaining isolates were VanA genotype (Nelson et al., 2000). Although the prevalence of vancomycin resistance was low among the studied isolates, their pre-

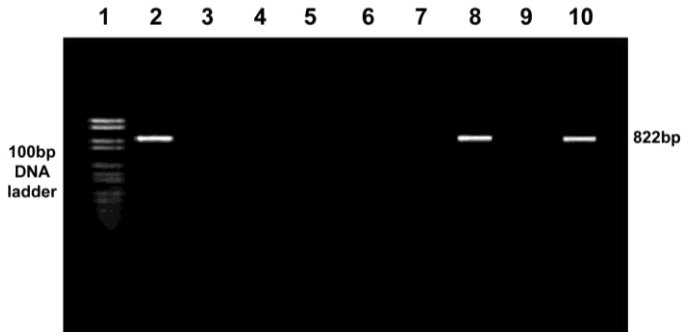

Figure 3. Agarose gel electrophoresis showing positive amplification of 822 base fragments specific for vanC of VRE from clinical specimens. Lane 1, size marker (100-bp DNA ladder); lane 2, *E. faecium* ATCC 29212 (positive control); lanes 3 to 7, *E. faecalis* VRE; lane 8 and 9, *E. faecium* VRE and lane 10 *E. gallinarum.* The figure shows that PCR of the VanC gene with a product size of (822 bp) was obtained from two isolates (one *E. gallinarum* specimen, and one *E. feacium* specimen).

sence together with high-level aminoglycoside resistance calls for regular surveillance studies, infection control and monitoring of antibiotic sensitivity. Presence of *vanA*, *vanB* and *vanC* gene cluster in some of the isolates can provide transfer of vancomycin resistance via conjugative plasmids not only to enterococci species but also to other bacteria such as *S. aureus.* So, we expect an increase in the number of VRE in the future.

Conclusion

This study shows an emergence of VRE along with increased rate of multidrug-resistant enterococci in cases of neonatal septicemia. Regular surveillance of antimicrobial susceptibilities should be done regularly and the risk factors should be determined.

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