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Isolation and species identification of enterococci from clinical specimen with their antimicrobial susceptibility pattern in a tertiary care hospital, Bangladesh

Tashmin Afroz Binte Islam[1*], S M Shamsuzzaman[2]

[1]*Department of Microbiology, Tairunnessa Memorial Medical College, Gazipur, Bangladesh*

[2]*Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh*

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ABSTRACT

Objective: To investigate the species prevalence of *Enterococcus* with their antimicrobial resistance pattern from patients of Dhaka Medical College Hospital.

Methods: Samples were cultured and *Enterococcus* species were identified by conventional biochemical tests as well as PCR by using species specific primers for *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*). For isolation of vancomycin resistant enterococci, minimum inhibitory concentration of vancomycin and PCR was done to detect *vanA* and *vanB* genes.

Results: A total of 16 enterococci were isolated from 300 urine and 200 wound swab samples (15 from urine and 1 from wound swab) from July 2011 to June 2012. Enterococci were the third most common organism (8.47%) from urine after *Escherichia coli* (63.28%) and *Enterobacter* (11.87%). Out of 16 enterococci, 10 (62.5%) were *E. faecalis*, 4 (25%) were *E. faecium* and 2 (12.5%) were other species. All the enterococci (100%) were sensitive to vancomycin and linezolid. Most of the strains were resistant to ciprofloxacin and azithromycin (87.5%), gentamycin (81.25%), ceftriaxone (75%), amoxiclav (31.25%) and imipenem (25%). *E. faecium* was more resistant than *E. faecalis* to azithromycin (100%), ciprofloxacin (100%), amoxiclav (75%) and imipenem (50%). No vancomycin resistant enterococci were identified and the range of minimum inhibitory concentration for vancomycin was 1–4 μg/mL. None of the enterococci were positive for *vanA* and *vanB* genes.

Conclusions: The presence of multidrug resistant enterococci should be considered as danger alarm for serious enterococcal infections and further study in large scale is needed.

1. Introduction

Enterococci are the member of normal flora in the gut of humans and animals[1]. Though they are not considered to be highly virulent, their intrinsic resistance and ability to acquire resistance to several broad spectrum antibiotics allow them to cause superinfections in patients already receiving antimicrobial therapy. *Enterococcus* species are most commonly implicated with infections of urinary tract, gastrointestinal tract, post surgical wounds, septicemia, endocarditis and meningitis[1].

Enterococcus faecalis (*E. faecalis*) is the most commonly isolated pathogen, followed by *Enterococcus faecium* (*E. faecium*) which are responsible for most of the human infections. Species identification is useful for epidemiological investigation of an outbreak and also for clinical decisions, particularly with regard to therapy, as antimicrobial susceptibility differs by species[2]. Biochemical tests for species identification are not performed routinely as they are laborious and time consuming; so to overcome the problem, the use of molecular methods has been suggested[3].

Enterococci have a tremendous capacity to acquire high level of resistance to penicillins, aminoglycosides and vancomycin making the treatment options limited for clinicians[2]. Vancomycin resistant enterococci have been reported worldwide and have caused hospital outbreaks[4-6]. The incidence of enterococcal infections and species prevalence in Bangladesh are not thoroughly investigated.

The present study was undertaken with the objective of isolation, species identification of enterococci from clinical specimens, to

*Corresponding author: Tashmin Afroz Binte Islam, Assistant Professor, Department of Microbiology, Tairunnessa Memorial Medical College, Gazipur, Bangladesh.
Tel: +8801749071601
E-mail: dr.tasminsomc@gmail.com

determine the antimicrobial susceptibility pattern of the isolates and to detect vancomycin resistant enterococci phenotypically and genotypically.

2. Materials and methods

2.1. Samples

A total of 300 urine and 200 post operative wound swab samples were collected from patients of Dhaka Medical College Hospital of Bangladesh, over 12 months period from July 2011 to June 2012. Data regarding age and sex were recorded.

2.2. Ethical issue

Approval was obtained from Research Review Committee and Ethical Review Committee of Dhaka Medical College according to the Declaration of Helsinki and national and institutional standards. Informed written consent was obtained from all participants before collecting samples.

2.3. Isolation of enterococci

The samples were cultured on blood agar and MacConkey agar media and incubated at 37 ˚C for 24 h aerobically. Enterococci were identified by colony morphology, Gram staining, absence of motility and catalase production, tolerance to 6.5% NaCl, ability to grow at 10 ˚C and 45 ˚C, growth on bile esculin agar with esculin hydrolysis[7].

Species identification was done by fermentation of mannitol, sorbitol, raffinose and arabinose, pyruvate utilization and arginine decarboxylation tests[8]. PCR was done to detect *E. faecalis* and *E. faecium* by using species specific primers[9].

2.4. Antimicrobial susceptibility testing

Standard disc diffusion techniques as recommended by the Clinical and Laboratory Standard Institute were performed for susceptibility testing of ciprofloxacin, ceftriaxone, gentamicin, azithromycin, amoxiclav, imipenem, vancomycin, linezolid (Oxoid, UK)[10]. *E. faecalis* ATCC 29212 strain was used as quality control.

2.5. Detection of vancomycin resistant enterococci

For detection of vancomycin resistant enterococci and minimum inhibitory concentration (MIC) of vancomycin, disc diffusion method and agar dilution method were done. An inhibitory zone diameter of 14 mm around vancomycin disc (30 μg) was considered as resistant, inhibitory zone of 15–16 mm was considered as intermediate and 17 mm was considered as sensitive. Similarly, MIC of 4 μg/mL for vancomycin was considered as sensitive, 8–16 μg/mL was considered as intermediate and 32 μg/mL was considered as vancomycin resistant enterococci[10]. Specific primers were also used for detection of *vanA* and *vanB* genes by PCR[9].

2.6. Determination of MIC

Five hundred milligrams base of commercially available vancomycin injection vial was added to 50 mL distilled water to a concentration of 10 mg/mL. For each plate, 50 mL sterile Mueller-Hinton agar was prepared and impregnated with 2.5 μL, 5 μL, 10 μL, 20 μL, 40 μL, 80 μL and 160 μL of vancomycin stock solution to achieve a concentration of 0.5 μg/mL, 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL and 32 μg/mL per plate, respectively.

2.7. DNA extraction

DNA was extracted by using boiling method. Bacterial colonies were suspended in 300 μL of distilled water and boiled for 10 min in a heat block, then placed on ice for 5 min. After centrifugation at 13 000 r/min at 4 ˚C for 5 min, the supernatant was taken in a micro tube and was kept at 4 ˚C until used as DNA template[11].

2.8. Amplification of *E. faecalis*, *E. faecium*, *vanA* and *vanB* genes

E. faecalis, *E. faecium*, *vanA* and *vanB* genes were amplified by using the primers (Table 1), as described previously[9]. The multiplex PCR assay was performed in a total volume of 25 μL containing 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.25 mmol/L each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 1 IU of *Taq* DNA polymerase (Promega Corporation, USA). DNA amplification was carried out with the following thermal cycling profile: initial denaturation at 95 ˚C for 10 min, 35 cycles of amplification (denaturation at 95 ˚C for 45 s, annealing at 54 ˚C for 1 min, and extension at 72 ˚C for 1 min), and a final extension at 72 ˚C for 10 min in a thermal cycler (Eppendorf AG, Mastercycler gradient, Germany). PCR products were analyzed on a 1.5% agarose gel with 0.53 Tris-borate-EDTA buffer. A 100-bp DNA ladder (Promega Corporation, USA) was used as the molecular size marker. The gels were stained with 1% ethidium bromide and visualized under UV light[9].

Table 1
Primers used in this study.

Genes	Sequence (5'-3')	Reference	Product size (bp)
E. faecalis	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	10	941
E. faecium	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	10	658
vanA	ATGAATAGAATAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	10	1 030
vanB	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	10	433

3. Results

A total of 16 enterococci were isolated from urine and wound swab with the prevalence of 8.47% in urine and 0.58% in wound swab. *E. faecalis* were the most frequently identified *Enterococcus* species (62.5%), followed by *E. faecium* (25%) and other *Enterococcus* species (12.5%). All the 10 *E. faecalis* and 4 *E. faecium* identified

by biochemical tests were positive by PCR using species specific primers (Figures 1 and 2).

In the present study, the *Enterococcus* isolates showed the highest rate of resistance in case of azithromycin, gentamycin and ciprofloxacin (Table 2). *E. faecium* exhibited higher rate of resistance to antimicrobial agents than *E. faecalis*. None of the isolates were resistant to vancomycin and linezolid. The MIC of vancomycin of 6 (37.5%) enterococcal isolates had 1 μg/mL, 7 (43.75%) had 2 μg/mL and 3 (18.75%) had 4 μg/mL. None of them were positive for *vanA* and *vanB* genes.

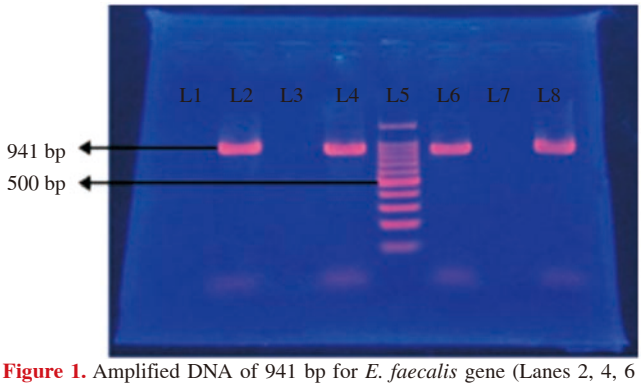

Figure 1. Amplified DNA of 941 bp for *E. faecalis* gene (Lanes 2, 4, 6 and 8) among enterococci isolated from urine.
Lane 5: Hundred base pair DNA ladder.

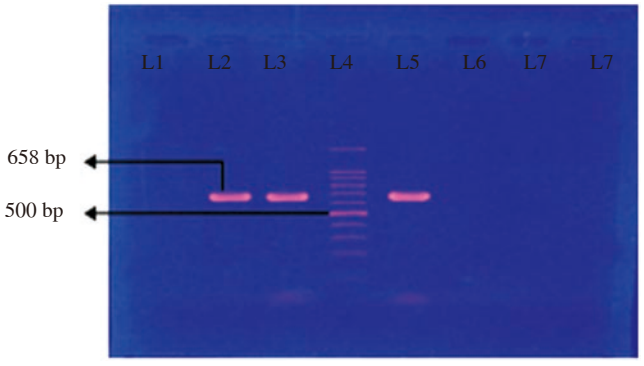

Figure 2. Amplified DNA of 658 bp for *E. faecium* gene (Lanes 2, 3 and 5) among enterococci isolated from urine.
Lane 4: Hundred base pair DNA ladder.

Table 2
Antimicrobial resistance pattern of *Enterococcus* species. *n* (%).

Antimicrobial agents	*Enterococcus* species	*E. faecalis*	*E. faecium*	Other species
Azithromycin	14 (87.50)	9 (90.00)	4 (100.00)	1 (50.00)
Ciprofloxacin	14 (87.50)	8 (80.00)	4 (100.00)	2 (100.00)
Gentamycin	13 (81.25)	10 (100.00)	2 (50.00)	1 (50.00)
Ceftriaxone	12 (75.00)	9 (90.00)	3 (75.00)	0 (0.00)
Amoxiclav	5 (31.25)	2 (20.00)	3 (75.00)	0 (0.00)
Imipenem	4 (25.00)	2 (20.00)	2 (50.00)	0 (0.00)
Linezolid	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Vancomycin	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

Enterococcus colonization was highest (68.75%) after 40 years of age. The enterococcal infection was 31.25% in male and 68.75% in female (Table 3). *Escherichia coli* (*E. coli*) was the most commonly isolated bacteria from urine (63.28%) and wound swab (36.42%) (Table 4).

Table 3
Age and sex distribution of 16 *Enterococcus* culture positive cases. *n* (%).

Age group in years	Male	Female	Total
0–20	0 (0.00)	1 (6.25)	1 (6.25)
21–40	1 (6.25)	3 (18.75)	4 (25.00)
41–60	2 (12.50)	4 (25.00)	6 (37.50)
⩾ 60	2 (12.50)	3 (18.75)	5 (31.25)
Total	5 (31.25)	11 (68.75)	16 (100.00)

Table 4
The prevalence of other organisms along with enterococci isolated from urine and wound swab. *n* (%).

Species of bacteria	Urine	Wound swab
E. coli	112 (63.28)	63 (36.42)
Enterobacter spp.	21 (11.87)	4 (2.31)
Acinetobacter baumannii	10 (5.65)	9 (5.20)
Klebsiella pneumoniae	7 (3.96)	17 (9.83)
Staphylococcus aureus	3 (1.69)	44 (25.43)
Pseudomonas aeruginosa	3 (1.69)	15 (8.67)
Proteus spp.	2 (1.13)	11 (6.36)
Citrobacter freundii	2 (1.13)	2 (1.15)
CoNS	2 (1.13)	7 (4.05)

CoNS: Coagulase negative *Staphylococcus*.

4. Discussion

Nosocomial infections with enterococci are a major concern at many hospitals throughout the world including hospitals in Bangladesh[12]. The epidemiology of enterococci is not fully understood since there are striking differences among different species of resistant isolates obtained from various geographic locations[13].

In the present study, the prevalence of enterococcal urinary tract infection (UTI) was 8.47% which correlates with the findings of other authors who found that 8.2% and 6.8% of the urine cultures were positive for enterococci respectively[14,15]. One study from Bangladesh reported that *Enterococcus* was the third most frequent cause of UTI after *E. coli* and *Enterobacter* which is similar to our study[16].

Studies carried out in Gaza reported 1.9% enterococci in wound swab but in present study only one (0.58%) *Enterococcus* was found in wound swab; this may be due to the incidence of infection varying from place to place and country to country due to different therapeutic and preventive policies[17].

In this study, higher proportions of *Enterococcus* positive patients (68.75%) were > 40 years of age and not infrequent in less than 20 years of age which supports other study[18]. The reason of higher infection rate among elderly people might be due to the fact as age advances people are more exposed to external environment and many of them might have history of taking treatment from different health care facilities, which might serve as a source of transmitting this infection. Moreover, immunity decreases with the advance of age which may help in colonization of these bacteria. In present study, female (68.75%) are more infected than male (31.25%) which is similar with the report of another study[2]. In Bangladeshi community, females are neglected and usually they take treatment from government facilities where treatment cost is very low and this might be the reason of getting higher enterococci infection rate among females in the present study. Moreover, anatomically females are more prone to develop UTI than males.

In present study, among 16 enterococci, 10 (62.5%) were identified as *E. faecalis* and 4 (25%) as *E. faecium* which is similar to another report[19]. The prevalence of *E. faecalis* over *E. faecium* in causing infection may be due to enhancing virulence traits of *E. faecalis*[20].

Resistance to a number of antimicrobial drugs is a characteristic of the genus *Enterococcus*. In the present study, *Enterococcus* isolates showed high level of resistance to ciprofloxacin (87.5%), azithromycin (87.5%), gentamycin (81.25%), and ceftriaxone (75%) which coincides with the previous studies[16,21,22].

In this study, *E. faecium* was found more resistant to many antibiotics than *E. faecalis,* which is similar with the reports of other studies[20,21]. We found that (100%) *E. faecium* were resistant to ciprofloxacin, 75% to amoxiclav, and 50% to imipenem, on the other hand *E. faecalis* showed 80% resistance to ciprofloxacin, 20% to amoxiclav and imipenem. Due to the variation in resistance pattern in different antibiotics for different species of *Enterococcus*, identification of species is important for proper treatment.

In present study, no *Enterococcus* was found resistant to vancomycin and linezolid and the MIC range of vancomycin was 1–4 μg/mL which correlates with the other reports[19-21].

All the *E. faecalis* and *E. faecium* identified by biochemical tests showed positive result in PCR. As the identification of species by biochemical tests is a tedious process that requires numerous tests and long time, the use of molecular techniques could therefore enhance the identification process.

In conclusion, it was shown that enterococci are the third leading cause of UTI and resistant to multiple antibiotics and vancomycin and linezolid may be the drug of choice to treat these infections. Regular susceptibility test and identification at species level are important in order to treat enterococcal infections effectively and implement appropriate infection control measures to limit the spread in nosocomial settings.

Conflict of interest statement

We declare that we have no conflict of interest.

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