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Species prevalence, antimicrobial susceptibility and detection of virulence factors of enterococci isolated from tertiary care hospital

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Abstract---Introduction: Emergence of multidrug resistant nosocomial enterococcus strains emphasizes the need for further investigating enterococci. Objectives: To characterize enterococci from various clinical specimens, to determine the antimicrobial susceptibility pattern and to explore the association between virulence factors and antimicrobial resistance. Material and Methods: Two hundred and eighty three clinical isolates of enterococcus were speciated and subjected to antimicrobial susceptibility testing using Kirby Bauer disc diffusion method. They were screened for vancomycin resistance by vancomycin screening agar method as recommended by Clinical Laboratory Standards Institute 2014, and confirmed by determination of minimum inhibitory concentration using agar dilution and E test. Genotypic confirmation was done by polymerase chain reaction. Virulence factors (haemolysin, gelatinase and biofilm production) were detected phenotypically. Results: Of the 283 enterococci isolated, 12 species were identified; predominant species was *Enterococcus faecalis* (82.33%). High level gentamicin resistance (HLGR) and vancomycin resistance were observed among 55.57% and 6.01% of *Enterococcus* isolates respectively. All vancomycin resistant enterococci (VRE) were *Enterococcus faecalis* and had vanA phenotype and genotype. Sensitivity to linezolid was 100 per cent among enterococci. Hemolysin, gelatinase and biofilm production were seen in 15.90%, 12.36% and 13.43% of enterococcal isolates respectively. HLGR and vancomycin resistance were observed in 61.86% and 0.35% of the 118 isolates producing virulence factors. Conclusion: Our study reveals the occurrence of sizable number of HLGR isolates and emergence of VRE. Isolates resistant to HLG but susceptible to vancomycin expressed more virulent factors. Further research is required to reveal the complex interplay between drug resistance and virulence factors.

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Keywords---antimicrobial sensitivity, enterococcus, virulence factors, VRE.

Introduction

Enterococci, recognized as opportunistic pathogens, are natural inhabitants of the oral cavity, gut and the female genital tract in both humans and animals[1]. They are traditionally regarded as low grade pathogens, have emerged as an increasingly important cause of nosocomial infections in the last decade. Although about a dozen enterococcus species have been identified, only two are responsible for the majority of human infections, *i.e. Enterococcus faecalis* and *E. faecium*[2]. Nevertheless, incidence of other species of enterococci from clinical sources shows an alarming increase with properties of intrinsic resistance to several antibiotics[3]. Until recently, vancomycin was virtually the only drug that could be consistently relied on for the treatment of infections caused by multidrug-resistant enterococci[4]. However, emergence of vancomycin resistant enterococci (VRE) and their increasing prevalence worldwide has made it difficult to treat serious enterococcal infections[2]. Along with emergence of multidrug resistance, presence of several virulence factors in enterococci is an emerging concept. Few species like *Enterococcus gallinarum* and *E. casseliflavus* are intrinsic resistant to vancomycin so it becomes essential to identify these species in order to avoid inappropriate treatment with vancomycin. Hence, Knowledge of the profile of enterococcal species, their antimicrobial resistance and associated virulence factors is quintessential for management and prevention of these bacteria in any healthcare facility and to formulate treatment guidelines for infections caused by enterococci. Knowing the paucity of data on enteroccous infection from Kumaon region this study was undertaken.

Materials and Methods

All consecutive strains of enterococcus isolated from various clinical samples received over a period of two years were identified and speciated according to standard laboratory procedure as per the scheme of Facklam and Collins[5]. For studying the antimicrobial susceptibility pattern in enterococcal isolates, four methods were used a) Kirby-Bauer disc diffusion technique[6], b) vancomycin screening agar method[6], c) minimum inhibitory concentration (MIC) testing by E strips and d) agar dilution method[7] for the enterococci isolates which were found resistant on vancomycin agar screening plate.

Determination of antimicrobial susceptibility by Kirby-Bauer disc diffusion method

For disc diffusion testing the following antimicrobial discs and concentrations were used: Ampicillin (10µg), High level gentamicin (120µg), Erythromycin (15µg), Vancomycin (30µg), Teicoplanin (30µg) and Linezolid (15µg). For urine isolates antimicrobial discs of Ampicillin (30µg), High level gentamicin (120µg), Levofloxacin (5µg), Norfloxacin (10µg), Nitrofurantoin (300µg), Vancomycin (30µg), Teicoplanin (30µg), and Linezolid (30µg) were used.

Vancomycin screen agar test

Vancomycin screen agar test was performed using brain heart infusion (BHI) agar with 6 µg/ml vancomycin to look for resistance to vancomycin[6]. Control strain included were *E. faecalis* ATCC 29212 and *E. faecium* ATCC 51559.

Determination of Minimum inhibitory concentrations by Vancomycin agar dilution method

Isolates showing growth on vancomycin agar-screening were further subjected for MIC of vancomycin by vancomycin agar dilution method as recommended by CLSI guidelines[7]. The minimum concentration of vancomycin which inhibited bacterial growth was considered MIC. Enterococci which had MIC >32 µg/ml were considered resistant; 8-16 µg/ml as intermediately resistant and MIC of 4 µg/ml as susceptible to vancomycin[7].

Genotypic characterization of vancomycin resistance

Simple PCR were performed for detection of VanA gene among VRE isolates. Briefly, the 25 µl of PCR contained; 2-4 well isolated colonies, 2.5 µl, 10× PCR buffer, 2 µl, 25Mm MgCl2, 1µl, 10Mm dNTPs, 1µl, 10Pm forward primer (5'GCGATATTCAAAGCTCAGCAA 3') 1µl, 10Pm reverse primer (5'TGCCGATTCAATTGCGTAGTC 3'), 0.5µl Taq DNA and 17µl nuclease free water. Reaction were performed in thermocycler, initial denaturation was done at 94°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 51.70 °C for 1 minute, extension at 72 °C for 1 minute and final extension done at 72 °C for 7 minutes. Amplified PCR products were detected by 1.5% agarose gel electrophoresis[8].

Phenotypic detection of virulence factors

Virulence factors (hemolysin, gelatinase and biofilm production) were detected phenotypically. Haemolysin production was detected by 5% blood agar[9]. Gelatinase activity was detected in 4% gelatin agar and clearing seen by mercuric chloride solution[9]. Biofilm formation was detected by tube method[10].

Results

A total of 283 enterococcus strains were isolated from different clinical samples during the period two years from November 2013 to October 2015. The sources and the species of 283 enterococcal isolates are summarized in Table 1. Of the 283 enterococci isolates, 12 species were identified; predominant species was *Enterococcus faecalis* (82.33%) followed by followed by *E. hirae* 10 (3.53%), *E. dispar* 09 (3.18%), *E. durans* 07 (2.47%), *E. asini* 04 (1.41%) and *E. faecium* 03 (1.06%). *E. faecium* was isolated only from urine samples.

Antimicrobial susceptibility testing

Of the 283 isolates, 55.57% were high level gentamicin resistant, 82% were resistant to erythromycin, 54.77% to ampicillin, 6.01% to vancomycin and

teicoplanin. Among urinary isolates maximum resistance was seen against norfloxacin (87.93%), followed by minocycline (76.70%), HLG (55.47%), ampicillin (54.77%), levofloxacin (49.13%), nitrofurantoin (26.72). Vancomycins resistant in 6.01% isolates were confirmed by disc diffusion, agar screen method and MIC (agar dilution and E-test). All VRE isolates had MIC >256 µg/ml, both by E-test and agar dilution method. No resistance was seen against linezolid (Table 2). All 6.01% VRE were identified as *Enterococcus faecalis* from in-patients and among them highest number were isolated from blood sample and lowest from urine sample.

Genotypic characterization of vancomycin resistance

A total of 17 VRE confirmed phenotypically as Van A were further confirmed by PCR Van A genotype. One PCR product was sequenced and confirmed the gene. The GenBank accession number assigned to our sequence is KU 667286.

Virulence factors produced among enterococcal isolates

Among virulence factors tested, hemolysin was produced by 15.90% of enterococcal, biofilm by 13.43% and gelatinase by 12.36% isolates. Species wise virulence factors production is depicted in Table 3.

Relationships between Enterococcal virulence and antimicrobial resistance

Of the 118 isolates, producing virulence factors, 73 (61.86%) were HLGR strains. Out of 45 hemolysin producing strains, 30 (66.67%) were HLGR. Similarly among gelatinase and biofilm positive strains more than 50% were HLGR. However, virulence factors were found more in ampicillin sensitive isolates as compare to resistant isolates, though this difference was not statically significant (Table 4). Out of 17 VRE isolates only one isolate was found to produce virulence factor i.e. gelatinase.

Discussion

Our observation concur with the other studies[11,12,13] which have found that 80 to 90% of clinical samples are *E. faecalis*. Nevertheless, the incidence of other species of enterococci from clinical sources shows an alarming increase[14]. Other researcher found greater proportion of *E. faecium* in blood cultures and *E. faecalis* in cultures of samples from other sites[15,16]. We found *E. faecium* isolates only from urine samples. A study from South India reported the prevalence of unusual (non-*faecalis* and non-*faecium* enterococci) and atypical (biochemical variant) species of enterococci as 19% (46 isolates) and 5% (12 isolates) respectively[1]. In present study, out of 283 enterococcus isolates, the biochemical phenotyping results revealed 47 isolates belonging to ten different unusual species of enterococci (excluding *E. faecalis* and *E. faecium*) which included 10 *E. hirae* (3.53%), 09 *E. dispar* (3.18%), 07 *E. durans* (2.47%), 04 *E. asini* (1.41%), 04 *E. cecorum* (1.41%), 03 *E. caccae* (1.06%), 02 *E. phoeniculicola* (1.06%), 02 *E. avium* (0.71%), 02 *E. italicus* (0.71%) and one *E. hermanniensis* (0.35%). Four species could not be identified due to aberrant sugar reactions by conventional method.

An interesting point of note was that *E. hirae* was the second commonest enterococcus species in the present study. *E. hirae* has been isolated only rarely in previous studies[17]. Recent literature shows a drastic increase in ampicillin resistance among enterococci[18]. In our study ampicillin resistance was observed in 54.77%. Similar results were obtained by other studies from North India[19,20]. In a study during 1989–1996, quite a low prevalence of HLGR (16%) was found[21]. However, the incidence of HLAR is increasing[13,20,22,23,24]. In present study HLGR was observed in 55.47% of enterococcal isolates. Similar resistance rates were reported in other studies from South India and North India[13,22,23,24]. In present study, ampicillin resistant was observed in 54.77% along with HLGR. Such a finding is of concern since HLAR aborates the synergistic antienterococcal effect of beta-lactam agents and aminoglycosides which is therapy of choice in enterococcal infection.

In the last two decades, the emergence of VRE and their increasing prevalence worldwide has made it difficult to treat serious enterococcal infections. The prevalence of VRE has been increasing in the past one decade in India [19,21], the prevalence of VRE has been reported to be between 0- 30 per cent[19,21,25,]. In the present study, vancomycin resistance was found among 6.01% of *Enterococcus* isolates, which is comparable with other studies in India[13,]. In present study, a total of 17 (6.01%) VRE were identified as *Enterococcus faecalis* and all were received from in-patients. However, other studies have observed higher rates of resistance to vancomycin among *E. faecium*[13,16]. Highest number of VRE in our study was isolated from blood sample and lowest from urine sample. All VRE in our study were multidrug resistant thus making the treatment in such cases extremely difficult. Linazolid demonstrated good entrococcal activity and may be kept as the drug of choice for vancomycin resistant isolates. However, the alarming finding of emergence of resistance to linezolid in *Enterococcal* isolates warrants judicious use of this drug in health care setting.

Various studies on virulence factors of enterococci have currently reported their widespread distribution. Banerjee *et al*[25] observed that 23%, 8%, 25% of *E. faecalis* isolates were haemolysin, gelatinase and biofilm producers respectively and 40%, 9.6%, 27% of *E. faecium* were haemolysin, gelatinase and biofilm producers respectively. In a recent study from South India, hemolysin production was seen in 82% of the clinical isolates, while gelatinase production was demonstrated in 40.6% of the isolates[13]. We observed that 15.9% of isolates were haemolysin producers, 12.36% were gelatinase producers and 13.43% were biofilm producers. Overall virulence factors were lower in *E. faecalis* 40% (95/233) as compare to other non faecalis species 46% (23/50). We observed that isolates resistant to HLG but susceptible to vancomycin expressed more virulence factors than vancomycin resistant ones. This is in congruence with the other studies from India. Since, the drug resistant determinants in enterococci and virulence genes are plasmid borne with immense ability for genetic exchange both intragenically and intergenically. It has been speculated that increase in one aspect of survival fitness reduces the other. Consequently acquisition of one set of plasmid may lead to loss of the other either due to incompatibility or due to fitness cost benefits. Another explanation of this aspect could be the cost of fitness of these emerging organisms, while emerging drug resistance in these isolates is sufficient enough for better survival, eliminating the requirement of

additional virulence. These speculations can only hold true when further research is done on these aspects of virulence in enterococci. Much remains to be revealed about survival and complex interplay between drug resistance and virulence factors,

Conclusion

Our study reveals the occurrence of sizable number of HLGR isolates and emergence of VRE. Linazolid demonstrated good entrococcal activity and may be kept as drug of choice among VRE isolates in our set up. Isolates resistant to HLG but susceptible to vancomycin expressed more virulent factors. Further research is required to reveal the complex interplay between drug resistance and virulence factors.

Table 1
Distribution of Enterococcus species in various clinical specimens

S.No	Species (n=283)	Urine	Pus	Blood	CSF	Bile	Peritoneal Fluid	Pleural Fluid
1	*E. faecalis* (n=233)	95	86	44	04	01	02	01
2	*E. hirae* (n=10)	06	01	02	-	01	-	-
3	*E. dispar* (n=09)	02	03	03	01	-	-	-
4	*E. durans* (n=07)	03	03	01	-	-	-	-
5	*E. asini* (n=04)	02	01	01	-	-	-	-
6	*E. cecorum* (n=04)	01	01	01	-	-	-	-
7	*E. caccae* (n=03)	01	02	-	-	-	-	-
8	*E. faecium* (n=03)	03	-	-	-	-	-	-
9	*E. phoeniculicola* (n=02)	02	-	-	-	-	-	-
10	*E. avium* (n=02)	-	02	-	-	-	-	-
11	*E. italicus* (n=02)	-	-	02	-	-	-	-
12	*E. hermanniensis* (n=01)	-	01	-	-	-	-	-
13	Unidentified (n=04)	01	02	01	-	-	-	-
	Total	116	102	55	05	02	02	01

Table 2
Antimicrobial susceptibility patterns of enterococci by Kirby- Bauer disc diffusion method

Antibiotic	No of sensitive isolates (%)	No of resistant isolates (%)
Ampicillin	128 (45.22)	155 (54.77)
HLG	126 (44.52)	157 (55.47)
Erythromycin	29 (17.36)	138 (82.63)
Nitrofurantoin	85 (73.27)	31 (26.72)
Norfloxacin	14 (12.06)	102 (87.93)
Levofloxacin	59 (50.86)	57 (49.13)
Minocycline	27 (23.27)	89 (76.70)

Vancomycin	266 (93.99)	17 (6.01)
Teicoplanin	266 (93.99)	17 (6.01)
Linezolid	283 (100)	0 (0)

Table 3
Species wise distribution of virulence factors among isolates

Species (no)	No. of enterococci producing hemolysin (%)	No. of enterococci producing gelatinase (%)	No. of enterococci producing biofilm (%)	No. of enterococci producing all three virulence factors hemolysin, gelatinase and biofilm (%)
E. faecalis (n=233)	35 (15.02)	28 (12.20)	32 (13.73)	05 (2.14)
E. faecium (n=03)	02 (66.67)	02 (66.67)	01 (33.33)	
Non- E. faecalis and non E. faecium (n=47)	08 (17.02)	05 (10.63)	05 (10.63)	02 (4.25)
Total (283)	45 (15.90)	35 (12.36)	38 (13.43%)	07 (2.47)

Table 4
Relationships between Enterococcal virulence and antimicrobial resistance

Virulence factors	HLG Resistance	HLG Sensitive	Total no.
Haemolysin	30	15	45
Gelatinase	23	12	35
Biofilm	20	18	38
Total	73	45	118
Chi-sq value is 2.033, df=2, p is 0.0.362			
Virulence factors	Ampicillin resistance	Ampicillin sensitive	Total no.
Haemolysin	18	27	45
Gelatinase	13	22	35
Biofilm	18	20	38
Total	49	69	118
Chi-sq value is 0.854, df=2, p is 0.652			
Virulence factors	Vancomycin resistance	Vancomycin sensitive	Total no.
Haemolysin	0	45	45
Gelatinase	01	34	35
Biofilm	0	38	38
Total	01	117	118

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