A Clinicomicrobiological Study of Enterococcus Species with Special Reference to Its Virulence Factors

Sharma Revati, Pai (Bhat) Chitra, Urhekar A.D.

Department of Microbiology, MGM Medical College, Navi Mumbai, Maharashtra, INDIA.

Corresponding Address:

revupattani@gmail.com

Research Article

Abstract: Clinical samples were screened for enterococcus species. 215 enterococcal isolates were biochemically characterized. Seven species of enterococcus could be identified. The isolates were further screened for the presence of virulent factors such as hemolysin, gelatinase, enterococcal surface protein (esp) and aggregation substance (Agg) both phenotypically and genotypically. Virulent factors are known to cause an increase in the severity of the infection in animal models. The isolates produced 54 (25%), 77 (36%), 122 (57%) and 2(1%) of hemolysin, gelatinase, enterococcal surface proteins and aggregation substance respectively. The hemolysin (cylA) and gelatinase (gelE) genes did not correlate with their respective phenotypic expression indicating the presence of silent genes. The urine isolates had the maximum number of virulence factors.

Keywords*:* Enterococcus species, clinical infections, virulence factors.

Introduction

Enterococci are a part of the normal intestinal flora of humans and animals. They are gram positive non spore forming organisms usually inhabiting the alimentary canals of humans in addition to being isolated from environmental and animal sources. Earlier classified as Group D streptococci, they have now been placed under a separate genus. This genus at present includes more than 47 species. Most infections caused by enterococci in the past were associated with Enterococcus faecalis and Enterococcus faecium. [(1)] However in recent times, there has been an alarming increase in the emergence of unusual species of enterococci with intrinsic resistance to several antibiotics including beta lactams and glycopeptides. Most of the laboratories around the world only identify the organism upto the genus level. Identification of enterococcal isolates to the species level in the clinical microbiology laboratory is useful because it can help predict patterns of antimicrobial susceptibility. The spread and the mechanism of acquisition of drug resistance have been widely studied but enterococcal virulence and pathogenic mechanisms are largely unknown.[(2)]Previous studies have analyzed the distribution of virulence factors but none of them have focused on pathogenic strains isolated from significant infections. Hence a detailed study of the virulence factors can lead to a better understanding of the pathogenesis of enterococcal infections. Such virulence factors may play an important role in enhancing the pathogenicity and are expected to be associated with infections with a higher degree of severity as well as with nosocomial or hospital acquired infections. A number of studies have identified different virulence factors in enterococci. The most important among them are hemolysin, gelatinase, enterococcal surface protein [esp], aggregation substance [AS], MSCRAMM Ace (Microbial surface component recognizing adhesive matrix molecule adhesin of collagen from enterococci), serine protease, capsule, cell wall polysaccharide and superoxide. Although these have been identified since a long time, there haven't been much reports related to the association between the virulence factors and the different types of infections caused by Enterococcal spp. Region wise studies focusing on various enterococcal infections would help generate data regarding the prevalence of strains equipped with virulence factors which can also be correlated with their higher degree of pathogenicity and propensity to develop multidrug resistance. Paucity of such data from Navi Mumbai prompted us to undertake such a study in a tertiary care hospital.

Materials and methods:

Over a period of two years, from 6731clinical samples processed in the Microbiology laboratory, 215 strains of enterococci were isolated. The samples included blood, urine, catheter tube tip, sputum, vaginal swabs, body fluids, endotracheal tube secretions and pus. The isolates were further identified as follows:

I) Presumptive identification:

Samples were cultured on to5 % blood agar and Mac Conkeys agar. Gram positive cocci showing negative catalase reaction, positive PYR test, black

colonies on bile esculin agar and growth in 6.5% NaCl broth were presumptively identified as enterococci. Further, growth of the isolates at both 4^0C and 45^0C temperature confirmed them to be enterococci. Bacitracin sensitivity was also done to exclude other Streptococcus species

II) Characterization and speciation of the isolates:

The isolates which were primarily identified as enterococcus were then further characterized to the species level with the help of conventional biochemical methods as devised by Faclam and Collins [4]

This was based on:

a) Fermentation of the carbohydrates by using 1% solution of the sugars, glucose, lactose, raffinose, arabinose, sorbose, sucrose and sorbitol. b) Pyruvate utilization using 1% pyruvate slantc) Arginine decarboxylation using Moeller's decarboxylation brothd) Motility teste) Pigment production using nutrient agar.

For these biochemical tests, a single colony was picked and inoculated in brain heart infusion broth and incubated at 37^0C for 24 hrs. This was then used as an inoculum for the above tests. All the tests were incubated at 37^0C and read at 24 hrs.

III) Detection of virulence factors:

I) Phenotypic assays

a) **Hemolysin production:** The ability of the enterococcal isolates to produce hemolysin was analysed by hemolysin assay. [4]**.**The isolates to be tested were plated on blood agar base (Hi-media) supplemented with 5% human blood. The plates were incubated at 37^0C and observed after 24 hrs and 72 hrs. Plates were observed for alpha, beta and gamma hemolysis. Standard strains, E.faecalis FA 2-2 and E.faecalis JH2-2 were used as positive and negative controls respectively.

b) **Gelatinase production:** Gelatinase production was detected qualitatively by inoculating the clinical isolates onto gelatin agar (Hi-media) and incubating at 35^0C for 24 hrs as per standard method.[5] The growth of the isolates on the plate was then flooded with Frazier solution. A clear zone around the colonies indicated the digestion of gelatin and production of gelatinase by the organism. Standard strain used was E.faecalis MMH594 as positive control.

c) **Production of aggregation substance (AS) – Clumping assay:**

The assay as described by Roberta Creti et al, was used.[4]Briefly, 200µL of an 18 hour old culture supernatant of the pheromone producing E.faecalis JH2-2 strain grown in Brain heart infusion broth, was added onto each of the enterococcal strains being tested. After incubation at 37^0C for 24 hrs, Presence or absence of bacterial clumping was directly visualized and reported.

d) **Biofilm production**

The method of Jayanthi et.al was followed. [6] Biofilm was detected by inoculating the isolates into trypticase-soy broth [TSB] with 0.5% glucose and incubating at 37ºC. After overnight incubation, the culture was diluted 1:40 in fresh TSB-0.5% glucose. 200µl of the diluted solution was added to flat-bottomed polystyrene microtiter wells and incubated for 48 hours at 37ºC. Wells were gently washed three times with distilled water. After drying the plates in an inverted position at room temperature for 1 hour, the adherent biofilm was stained with 0.1% safranin and allowed to stand for 20 minutes at room temperature. Absorbance of the biofilm on the bottom surface of each well of the dried plates was determined at 490 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Test was carried out in triplicate and the average of the three optical density (OD) values was taken. Culture medium without organism was taken as blank. Biofilm producing *E. faecalis* OG1RF was taken as positive control. Mean OD value of positive control was taken as standard. Those values above 0.2 were considered as high biofilm producers. Values below 0.081 were categorized into non-biofilm producers. OD values above the standard but within 0.081 and 0.2 were taken as moderate biofilm producers.

II) Genotypic detection:

The virulence factors were genotypically determined by polymerase chain reaction method as per the procedure described by Eaton et al. [7]

The various genes amplified in the genotypic study of the virulence factors are as depicted in table 1.

Table 1: Virulence factors and their associated genes. [7]

Gene(s)	Role of gene in virulence	Size of the amplicon
Agg	Aggregation protein involved in adherence to eukaryotic cells; cell aggregation and conjugation	1553
gelE	Toxin; extracellular metalloendopeptidase, hydrolyzes gelatin, collagen, hemoglobin, and other bioactive compounds	419
cylA	Activation of cytolysin	517
Esp	Cell wall-associated protein involved in immune evasion; may be associated with *cyl*genes on a pathogenicity island.	933

a) DNA extraction and PCR assay.

DNA extraction and PCR was performed according to the procedure given by Brtkova A et.al [8] and Eaton et.al [7]) respectively. Template DNA was obtained by suspending overnight culture of isolates into the 100 µl STE (1M NaCl, 1M Tris, 0.5MEDTA, H_2O). Suspension was boiled for 10 minutes at 95°C and centrifuged for 5 min at 13 000 × g. The supernatant containing DNA was used as a template. PCR amplification was performed in 50µl reactions using 5 µg of DNA, 15 mM $MgCl_2$, 20 pmol of each primer and 1 U of DNA polymerase. Samples were then subjected to an initial cycle of denaturation (94°C for 2 min), annealing (at an appropriate temperature for 2 min), and elongation (72°C for 2 min), followed by 29cycles of denaturation (92°C for 15 s), annealing (at an appropriate temperature for 15 s), and elongation (72°C for 15 s).

b) **Detection of Amplicons.** This was carried out using agarose gel electrophoresis with 1.5% agarose gel using 1X Tris Acetate EDTA (TAE) buffer. A 100 base pair molecular weight marker (Pure gene) was used. The size of the desired amplicons have been described in Table 1

Results:

A total of 215 enterococcal strains were isolated from different clinical samples. 91 strains (42%) of enterococci were isolated from urine samples, 47 (22%) from pus, 21 (10%) from sputum, 18 (8%) each from blood and catheter tip and 9 (4%), 6 (3%), 3 (1%), 2 (1%) from endotracheal tip, pleural fluid, cerebrospinal fluid, and vaginal swabs respectively. Of the total 215 patients from whom the strains were isolated, 56% were from males and 44% were from females including newborns and children. Maximum number of enterococcal strains was isolated from urine samples (91%). The distribution of enterococcal strains isolated from various specimens is given in table 2

Table 2: Distribution of enterococci in clinical samples.

No of isolates Clinical Samples	E.faecalis	E.faecium	E.avium	E.raffinosis	E.casseli-Flavus	E.durans	E.gallinarum	Total
Urine	53 (58%)	33 (36%)	0	1 (1%)	2 (2%)	2 (2%)	0	**91(42%)**
Blood	6 (33%)	10 (56%)	1 (6%)	0	1(6%)	0	0	**18(8%)**
Catheter tip	4 (22%)	8(44%)	2 (11%)	3 (17%)	0	1 (6%)	0	**18(8%)**
Endotracheal tube	7 (78%)	1(11%)	0	0	0	1(11%)	0	**9(4%)**
Pus	23(49%)	10(21%)	5(11%)	3(6%)	2(4%)	2(4%)	2(4%)	**47(22%)**
Vaginal Swab	1(50%)	1(50%)	0	0	0	0	0	**2(1%)**
CSF	3 (100%)	0	0	0	0	0	0	**3(1%)**
Pleural fluid	3(50%)	2(33 %)	1(17%)	0	0	0	0	**6(3%)**
Sputum	13(62%)	5(24%)	2(10%)	1(5%)	0	0	0	**21(10%)**
Total	**113(53%)**	**70(33%)**	**11(5%)**	**8(4%)**	**5(2%)**	**6(3%)**	**2(1%)**	**215**

Following characterization of the strains, the most predominant species identified was Enterococcus faecalis (113,53%) followed by Enterococcus faecium (70, 33%). In 32 cases (15%) unusual species of enterococci (non faecalis and non faecium) were identified which included; E. avium (11,5%), E. raffinosus (8,4%), E.casseliflavus (5,2%), E.durans (6,3%) and E.gallinarum (2,1%).E.faecalis was further subspeciated and it was found that amongst the E.faecalis strains, E.faecalis var.liquefaciens (52, 45%) was the most prevalent followed by E.faecalis var. haemolyticus (16,14%), and E.faecalis var. zymogens (7,6%). (Table 3)

Table 3: Differentiation of subspecies of E.faecalis.

E.liquefaciens	E.faecalis var hemolyticus	E.faecalis	E.zymogens
Gel +; Hae +	Gel-; Hem +	Gel- ;Hem+	Gel +;Hem +
52 (45%)	16 (14%)	38(33%)	7(6%)

E.faecalis outnumbered the other enterococcus spp in all the samples except in the case of blood samples as well as in catheter tip specimens where E.faecium was isolated in higher percentage. In the case of vaginal samples, both the species were isolated in equal numbers. (Table 2). Apart from 215 isolates which were identified appropriately by the biochemical characterization methods we found 8 atypical strains that showed aberrant

sugar reaction which include non lactose fermenting E.faecalis like species. Whole cell protein analysis can be done to validate the taxonomic status of the atypical strains.

Detection of virulence factors

Hemolysin production was detected in 54 (25%) of the 215 strains. Among these, 61% showed beta hemolysis and 39% produces alpha hemolysis. None were gamma hemolytic. Gelatinase production was detected in 77 (36%) of the 215 enterococcal isolates. Out of these, 52E.faecalis and 18 E.faecium strains were gelatinase producers. The results of production of biofilm helped categorize the isolates into low or weak, moderate and strong biofilm producers based on the approach of Upadhaya et.al.[1]Out of 215 isolates, 93 (43%) of the isolates non biofilm producers, 75 (35%) were moderate biofilm producers and 47 (22%) were strong biofilm producers. Amongst the clinical samples the highest number of biofilm producers were from urine samples 53 (43%) followed by 35 (29%) from pus samples. Out of all 215 enterococcal isolates, E.faecalis was the species which showed maximum biofilm production in 53%, followed by E.faecium in 32%.

The table 4 shows the prevalence of the four virulence factors among the clinical isolates of the present study.

Table 4: Prevalence of virulence factors in the clinical isolates.

		No (%) of isolates positive for virulence factors		
Organisms	**Virulence Factors**	**Blood**	**Urine**	**Exudates**
	(No. of positives)	**n=18**	**n=91**	**n=106**
E.FAECALIS (113)	BIOFILM (65)	3 (5)	34(52)	28(43)
	CLUMPING FACTOR (1)	-	1(100)	-
	HEMOLYSIN (30)	-	15(50)	15(50)
	GELATINASE (52)	3(6)	24(46)	25(48)
E.FAECIUM (70)	BIOFILM (39)	8(21)	16(41)	15(38)
	CLUMPING FACTOR (1)	-	1(100)	-
	HEMOLYSIN (14)	3(21)	7(50)	4(29)
	GELATINASE (18)	4(22)	10(56)	4(22)
OTHER ENTEROCOCCI (32)	BIOFILM (18)	-	3(17)	15(83)
	CLUMPING FACTOR (0)	-	-	-
	HEMOLYSIN (10)	-	4(40)	6(60)
	GELATINASE (7)	-	3(43)	4(57)

Among E.faecalis and E.faecium strains, the virulence factor which was maximally prevalent was the biofilm production followed by gelatinase. The highest prevalence of virulent factors was exhibited by the urine isolates. Similarly for the non faecalis – non faecium group, the highest prevalent virulent factors were biofilm and hemolysin and was maximally exhibited by the exudate isolates. However, production of aggregation substance was seen only in two out of 215 isolates which showed visible clumping in the presence of pheromone.

Fig 1 shows the frequency of number of virulence factors present in the clinical isolates of enterococci.

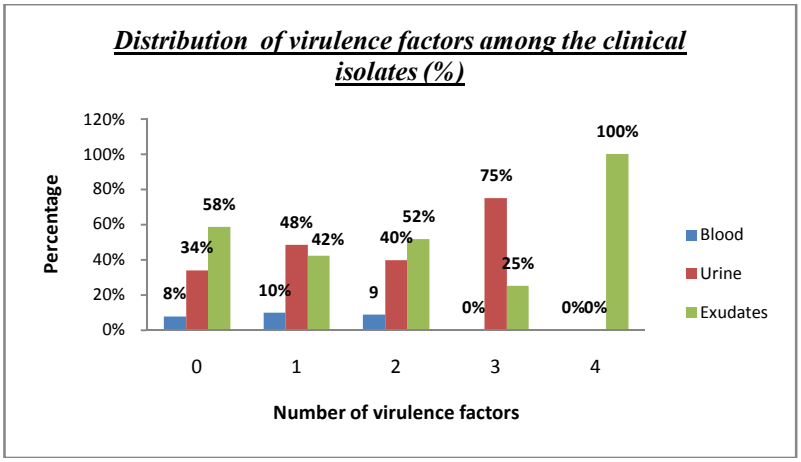

Fig 1: *Frequency of number of virulence factors presents in enterococci isolates.*

A comparison of occurrence of virulence factors phenotypically and genotypically was done and it was observed that the Agg and Esp genes were equally identified in phenotypic and genotypic assays however cyl A gene (hemolysin) was detected in 82 strains genotypically as compared to only 54 in phenotypic assay. Similarly for gelatinase, gel E gene was identified in 83 strains in comparison to 77 phenotypically gelatinase positive strains.

Discussion:

Enterococcus has emerged as an important nosocomial pathogen since the last decade worldwide[9, 10] The present study showed E. faecalis (53 %) and E faecium (33%) *as* the two .predominant species in our clinical setup accounting for (86%) of enterococci isolated. These findings were similar to other studies worldwide reflecting the general trend in prevalence of enterococci[9,11,12].Prevalence of unusual enterococcal species in 15% of the isolates was comparatively higher than the findings in other studies that showed a lower prevalence of 2-10% [9,13,14]. Some of the previous studies from India have only reported the prevalence of E.faecalis and E.faecium [15,16,17].From our perspective the prevalence tends to be higher which in part can be explained as misidentification of species due to exhibition of aberrant sugar reactions by some enterococci or due to lack of application of the complete range of tests to identify nonfaecalis and non-faecium enterococci on a routine basis in clinical diagnostics. [18]. The prevalence rate (15%) of our study was partly in accordance with another Indian study [19] that showed 14.8% as prevalence of unusual species of enterococci from catheterized patients with urinary tract infections. The highest prevalence rate of enterococci from urine samples is of concern because the urinary tract is the most common source of enterococci that cause bacteremia. [9] Among the UTI cases 76% cases were nosocomial acquired UTI while only 24 % were community acquired. In this study we have followed the classical approach for species identification of enterococci as suggested by Faclam and his colleagues. [3] Whole cell protein analysis can be done to validate the taxonomic status of the atypical strains. In this study the most prevalent virulence factor among the enterococci is the biofilm or enterococcal surface protein. In our study, the other less prevalent enterococci also produced Esp which contradicts the work from US and UK [20, 21].Our study was in accordance to a greater extent with a study conducted in Italy [22] wherein Esp or Biofilm was detected in 60% of the E.faecalis strains. However the study showed 72% of the E.faecium strains with the presence of Esp which is not the case in our report (56%). Our study showed that all the species apart from E.gallinarum and E.casseliflavus produced hemolysin which is in contradiction to several other studies showing that hemolysin is mostly produced by E.faecalis and E.faecium strains. Thus hemolysin production was found to a greater extent in our study. Our study depicted gelatinase production among 36% of the enterococci isolates. However many studies such as those of Kuhnen et.al [23] have reported a higher incidence of gelatinase production among the clinical isolates of enterococci in the order of 63.7 % isolates producing gelatinase. Although 6 isolates of E.faecalis showed clumping in the clumping assay only two of the isolates gave a true clumping reaction as Agg gene was present only in two of the isolates.

Conclusion:

Our study indicates the emergence of unusual species of enterococci and a change in the epidemiology of enterococcal infections in Western India. There is a need to study the prevalence of enterococcal species to reflect the exact local situation in different parts of our country. Several studies have reported the prevalence of very serious and life threatening enterococcal infections. Apart from looking at the drug resistance among these strains, it is also important to identify the virulence factors associated with the type of the disease. In depth study of virulence factors and their mechanisms would help develop strategies to suppress or inhibit them such that such infections can be contained better in the wake of development of high levels of drug resistance among the Enterococcus strains.

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