Detection Of Virulence Factors In Clinical Isolates Of Enterococci In A Tertiary Care Hospital Of Southern Maharashtra

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Abstracts: Background & Objective**:** Enterococcus is one of the notorious organism causing nosocomial infections. Several virulence factors have been described from enterococci that enhance their ability to colonize patient's tissue, increase resistance to antibiotics and aggravate the infection outcome. Therefore the study was carried out to investigate virulence factors among the enterococci isolated from different clinical samples. Methodology**:** Total 154 enterococcus species were isolated and were identified by conventional bacteriological method as well as by Vitek-2 compact system. The presence of certain virulence factors namely hemolysin, haemagglutination, gelatinase production and biofilm formation were detected and data was statistically analyzed. Results: *E. faecalis* was the commonest isolate (147) followed by *E. faecium* (7). Maximum number of isolates were from urine 65(42.20%) followed by pus 58(37.66%). The results of detection of virulence factors revealed 15.58% strains with gelatinase production, 34.41% with haemagglutination property, 35.71% showed hemolysin production and 55.84% strains showed biofilm production by microtitre assay plate method. Conclusion**:** All the markers studied are the potential virulence markers for the enterococci. Biofilm formation is the crucial virulence factor that enhances the ability of pathogen to cause severe infections. Inhibiting the action of such virulent factors or blocking biofilm formation may provide alternative method of therapy. [Patil S NJIRM 2016; 7(3):7-11]

Key Words: Biofilm, Enterococcus, virulence.

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Introduction: Enterococcus is one of the leading causes of nosocomial infection with *E. faecalis* and *E. faecium*. These bacteria frequently isolated from surgical wound infections, nosocomial bacteremia, endocarditis, urinary tract infections etc and are becoming resistant to multiple antibiotics. Because of this trend, treatment of enterococcal infection becomes complicated. Apart from this trend (which itself can be considered as a virulence factor) bacteria possesses many other virulence factors. Some of which are considered very important in pathogenesis and disease production. The virulence factors such as hemolysin, haemaglutination, gelatinase have been oftenly reported as potential virulence factors for enterococcus species [1]. Along with this, enterococci are also having ability to form biofilm [2]. Biofilm is a living ecosystem made of millions of adherent bacterial cell embedded within a self produced matrix of extracellular polymeric substance [2]. Biofilm producing bacteria are capable of adherence to damaged tissue and plastic surfaces such as medical devices. This helps bacteria for colonization and enhances capacity to cause infections [3]. It is an important virulent factor help to enhance survival of bacteria under adverse conditions and in presence of medicine.

By keeping all these things in mind the present research work was aimed to study enterococcus species from different clinical specimens with special emphasis on development of different virulence factor of the organism and their significance in clinical settings

Material and Methods:

Enterococcus species isolated from different clinical samples over a period of one year in the laboratory were included in the study. The strains were isolated from blood, pus, urine, body fluids etc. The isolates were identified up to genus and species level by gram stain, motility and a set of conventional biochemical tests such as, catalase test, growth in presence of 6.5% Nacl, esculin hydrolysis test, growth at 10^0C and 45^0C, arginine dihydrolase reaction, manitol, arabinose, lactose fermentation, etc by using standard recommended techniques [1]. Control strain used was *E. faecalis* ATCC *29212*. All the strains were retested by Vitek-2 compact system for species identification and antibiotic susceptibility testing as per manufacturer's instructions.

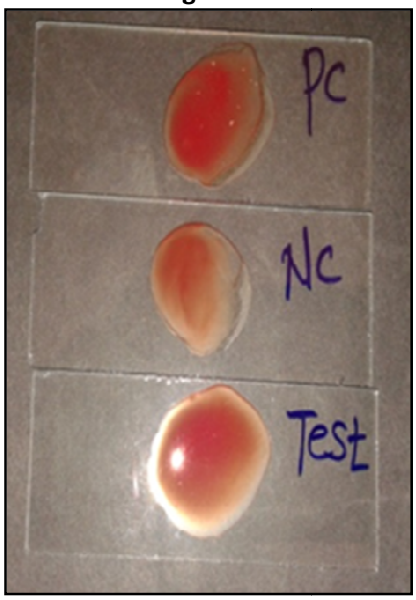
Detection of various virulence factors was done by using following phenotypic methods:-

1) Hemolysin assay: For detection of haemolysin, brain heart infusion agar (BHI) supplemented with 5% human blood was used. Agar plates were inoculated and incubated for 48 hrs, at 37^{0}C. β-hemolysis surrounding bacterial colonies was noted [1]. (Figure 1)

Figure 1

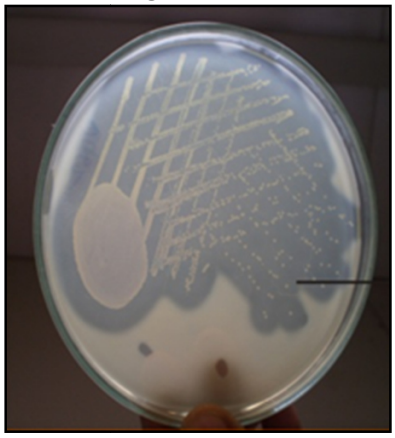
2) Haemagglutination assay: A drop of bacterial suspension prepared from growth on BHI agar supplemented with blood is mixed on a clean slide with 25µl of 3% human erythrocyte suspension in phosphate buffer saline. Results were noted after 5 minutes as positive or negative according to presence or absence of haemagglutination [1]. (Figure 2)

Figure 2



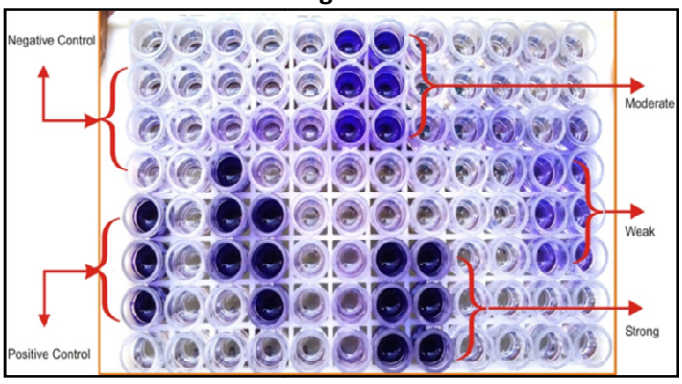
3) Gelatinase assay: Gelatinase activity was detected using gelatin agar (Hi Media). Plates were prepared and inoculated with fresh culture of isolates. These plates were incubated for 24 hrs at 37^{0}C. Gelatinase activity was observed as transparent halo around the colonies after flooding the plate with Frazier solution [4]. (Figure 3)

Figure 3



4) Microtitre plate assay for biofilm formation: Ability of the isolate to form biofilm on abiotic surface was quantified as per Toledo arena et al [3]. Briefly, overnight growth at 37^{0}C in BHI broth with 0.25% glucose was taken. Culture was diluted 1:20 in same media. 200 µl of this suspension was used to inoculate sterile 96 well polystyrene microtitre plates. After 24 hrs at 37^{0}C of static incubation wells are washed with phosphate buffer saline, dried in inverted position and stained with 1% crystal violet for 15 minutes. The cells were rinsed once more and the crystal violet was solubilized in 200 µl ethanol/acetone (80:20V/V). The optical density was determined using microtitre plate reader using A595 filter. Biofilm formation was scored as non biofilm forming (-), weak (+), moderate (++), strong (+++) corresponding to the A595 values ≤1, 1-≤2, 2-≤3 and 3 respectively [5]. The test was done in triplicates. *E. faecalis* OG1RF was taken as control. (Figure 4)

Figure 4



Statistical analysis: Statistical analysis was carried out using students paired and unpaired 't' test. P value less than 0.05 was considered as significant. (P < 0.05)

Results: Total 154 clinical isolates of enterococcal species from different clinical samples were tested for presence of virulence factors. Of the total isolates, 147 were *E. faecalis* and 7 were *E. faecium*. Maximum number of isolates were from urine (42.20%) followed by Pus (37.66%) and Blood (10.38%). (Table No. 1)

Table No 1: Sample wise distribution of enterococcal isolates

Sample	*E. faecalis* (n=147)	*E. faecium* (n=7)	Total (%)
Urine	64	1	65(42.20)
Pus	53	5	58(37.66)
Blood	15	1	16(10.38)
Vaginal swab	6	0	6(3.89)
Miscellaneous	4	0	4(2.59)
Fluid	3	0	3(1.94)
Catheter	2	0	2(1.29)

Table no 2: Virulence factors in clinical isolates of enterococcus

Virulence factor	*E. faecalis*	*E. faecium*	Total
Gelatinase	22 (14.96%)	2 (28.57%)	24 (15.58%)
Haemagglutination	50 (34.01%)	3 (42.85%)	53 (34.41%)
Hemolysin	54 (36.73%)	1 (14.28%)	55 (35.71%)
Biofilm	82 (55.78%)	4 (57.14%)	86 (55.84%)

Gelatinase production was seen in only 15.58% of clinical isolates tested. (Table No. 2) 11(7.14%) of urine isolates showed gelatinase activity while 10(6.49%) of the isolates from pus. In our study we found 34.41% strains showing haemagglutination production. (Table No. 2) Maximum number of isolates were from urine 27(17.53 %) followed by pus 15(9.74%). Of the 154 isolates 55 (35.71%) showed β-hemolysis on blood agar. (Table No. 2). Among the total 55 hemolytic enterocoocal isolates, 23(14.93%) were from urine and 22(14.28%) from pus. Biofilm production on microtitre plate was demonstrated by 55.84% of enterococcal isolates (Table No. 2). Even though, *E. faecalis* is predominant species isolated followed by *E. faecium* both were showed ability to form biofilm and did not differ significantly. (P=0.05). Total 41(26.62%) were showed weak biofilm production with values range between 1 to ≤2 on A595 filter. Whereas 29(18.83%) were moderate and 17 (11.03%) were strong biofilm producer with OD on A595 filter ranging from 2 to ≤3 and 3 respectively. (Table No. 3). 36 (23.37%) isolates from urine showed biofilm formation followed by pus 33 (21.42%)

Table no 3: Biofilm formation by enterococcus species from infectious sources on polysterene microtitre plates.

	(-) no biofilm	(+) weak	(++) moderate	(+++) Strong
Urine	30	16	13	06
Pus	23	21	06	08
Blood	06	04	05	01
Vaginal swab	04	--	01	01
Catheter	02	--	03	01
Fluids	02	--	01	--
Total (154)	67 (43.50%)	41 (26.62%)	29 (18.83%)	17 (11.03%)

Discussion: Enterococcus has attracted much attention in recent times due to their frequent association in nosocomial infections, especially in patients receiving antimicrobial agents [6]. Several virulence and pathogenicity factors have been described from enterococci which are helpful for them to colonize patient's tissue, and increase resistant to antibiotic and aggravate the infection outcomes [7]. In our study the expression of four important virulence factors such as hemolysin, gelatinase, haemagglutination and biofilm formation was evaluated.

Among all the virulent markers hemolysin is an important virulence marker in enterococcal infections[5]. Hemolytic activity was detected in 55(35.71%) of the strains. The ability to produce hemolysin helps these organisms to acquire adequate nutrition in the host tissue as well as further the spread of infection in the host body. Thus, it's production enhances severity of infection[8].

In the present study only 15.58% of the enterococcal isolates were gelatinase producer. These results are in

agreement with other studies which detect gelatinase production in range of 19%-31% among enterococci [9]. Gelatinase helps enterococci in providing nutrition by degradation of host tissues. The role of gelatinase causing endocarditis has been studied using animal models [9]. However, the low prevalence of this factor in our study suggests that this is probably having limited role in pathogenicity of enterococcal infection.

Agglutination of erythrocytes by enterococci is convenient major of adherence [10]. In the present study total 34.41% of strains showed haemagglutination. Haemagglutination by enterococcus species have been presumed to be caused by thermostable proteic compounds[10]. Our results showed that haemagglutination property is common for both the entercoccus species tested. Bacterial adhesion to host cell is recognized as the initial event of pathogenesis. Therefore, haemagglutination property might be playing pivotal role in establishment within host.

Total 82(55.78%) out of 147 *E. faecalis* strain and 4 (57.14%) out of 7 *E. faecium* were biofilm producers. (Table No. 2) There was no significant difference in the proportion of isolates producing biofilm. (P=0.05) Enterococci have been associated with biofilm on various kinds of indwelling devices and this capability to produce biofilm has been considered an important virulent factor. Increase ability to form biofilm in vitro is associated with medical device related infection in vivo [3]. Various methods have been tried to demonstrate this property.

The microtitre plate biofilm assay technique was found to be simple and cost effective. More than 50% of the strains revealed biofilm production by microtitre plate biofilm assay. The comparative study among the isolates with respect to the production of several virulence factors showed that biofilm production is high in numbers in all the clinical samples and it is very high among isolates grown from the cases of urinary tract infections and wound infections. It is evident from the study that biofilm formation is an important factor in enterococcal pathogenesis. Development of biofilm plays significant role in bacterial colonization during the infection and providing an opportunity for bacteria to develop antibiotic resistance [11]. Our results are agreed with previous studies, where they have demonstrated biofilm production and correlated with *esp* gene [2,11,12].

Conclusion: Our results indicate that all the virulent properties studied are more or less helpful for organism to establish and in disease production. Biofilm formation is crucial virulent property, as it helps bacteria to sustain and multiply in adverse environment. Therefore, there is increase in importance of enterococci in several diseases and its identity as a leading nosocomial pathogen. Blocking of biofilm production or by inhibiting the action of other virulence factors may provide alternative methods of therapy, which in turn will reduce use of antimicrobial agents and this will result in prevention of development of antimicrobial drug resistance among the enterococci.

References:

1. Jawad Kadhum T. AL-Khafaji, Sameer F. Saman, Mohammed S. AL-Saeed. Virulence Factors of *Enterococcus faecalis.* Medical Journal of Babylon 2010; 7 : 579-583
2. Ramadhan AA, Hegedus E. Biofilm formation and *esp* gene carriage in enterococci. J. Clinical Pathology 2005;58:685-687
3. Jonathan A.T. Sandoe, lan R. Witherden, Jonathan H. Cove, John Heritage and Mark H. Wilcox. Correlation between enterococcal biofilm formation in vitro and medical–device-related infection potential in vivo. Journal of Medical Microbiology 2003;52:547-550
4. Karola Waar, Albrecht B, Muscholl-Silberhprn, Rob J.L. Willems, Maarten J. H. sloff, Hermie J. M. et al. Genogrouping and Incidence of Virulence Factors of *Enterococcus faecalis* in Liver Transplant Patients Differ from Blood Culture and Fecal Isolates. JID 2002;185:1121-1127
5. R Arularasi Aberna, K Prabakaran. Evaluation for the association of virulence determinants among *E. faecalis* with its clinical outcome. Int J Biol Med Res. 2011;2(2):523-527
6. Giridhara Upadhyaya, P.M., Ravikumar, K.L., Umapathy, B.L. Review of virulence factors of enterococcus: An emerging nosocomial pathogen. Indian J. Med. Microbiol. 2009;27, 301-305.
7. Abbas A. Al-Duliami, Nadhum G. Nauman, Afak R. Salman, Abdul-Razak SH Hasan. Virulence factors of enterococci species isolated prom nosocomial and community acquired infections. Diyala journal for pure sciences.2011; 7 (3) : 174-82
8. Sanal C. Fernandes and B. Dhanashree. Drug Resistance and Virulence determinants in clinical

isolates of Enterococcus species. Indian J Med Res.2013;981 -985
9. Praharaj Ira, Sistla sujatha, Parija subhash Chandra. Virulence factors in Clinical and commensal isolates of Enterococcus species. Indian J pathol microbiol. 2013; 56(1) :24-30
10. Maria da Gloria S. carvalho, Lucia Martins Teixeira. Hemagglutination properties of Enterococcus. Current microbiology 1995; 30 : 265 – 268
11. Ajay Kumar oli, Raju S., Rajeshwari Nagaven. Biofilm formation by multidrug resistance Enterococcus faecalis (MDEF) originated from clinical samples. J Microbiol Biotech Res, 2012; 2(2): 284-288.
12. P. M. Giridhara Upadhaya, B L Umapathy and K. L. Ravikumar. Comparative study for the presence of enterococcal virulence factors gelatinase, hemolysin and biofilm Among clinical and commensal isolates of *Enterococcus faecalis*. J Lab Physicians.2010; 2(2): 100-104

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