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Original Research Article

Comparative Study for the Detection of Virulence Factors in Clinical and Commensal Isolates of Enterococcus Species

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ABSTRACT

Enterococcus, considered a normal commensal of intestinal tract is one of the fast emerging pathogen causing serious and life threatening nosocomial infections. Biofilm production, hemolysin and gelatinase are the potential virulence factors of Enterococci. The following study was carried out to evaluate few virulence determinants elaborated by strains of Enterococci in our setup and to ascertain if these strains differ considerably from the commensal strains of Enterococci.

Materials and Methods: One hundred and twenty isolates of Enterococcus species from clinical specimens and thirty isolates of Enterococcus species from stool samples were evaluated for the presence of virulence determinants like hemolysin, gelatinase and biofilm formation by phenotypic tests. Strains of Enterococcus from clinical samples and those present as commensals were compared with respect to virulence factors.

Statistical Analysis: Chi-square test carried out using SSPS Version 5.1 Software

Results: The clinical isolates produced 36.85%, 19.6% and 55.5% of hemolysin, gelatinase and biofilms, respectively as compared to 10, 6.7 and 26.6% produced by Commensal isolates, respectively.

Conclusion: Significant difference in the production of virulence factors ($p < 0.05$) was noted between clinical and commensal isolates .The virulence factors were more common in clinical isolates than commensal strains.

Keywords: Enterococcus, Biofilms, hemolysin, gelatinase, virulence factors, microtitre plate method.

INTRODUCTION

Enterococcus species have been considered as the commensals of gastrointestinal tract. But in the recent times the number of serious infections caused by these organisms has been steadily increasing. Enterococci rank among the leading causes of UTIs and Catheter associated endocarditis. This is attributed to the acquisition of Multidrug resistance and putative virulence factors. [1-3] A number of studies have identified different virulence factors, the most important among them being gelatinase, hemolysin, enterococcal surface protein (Esp), aggregation substance (AS), MSCRAAM Ace (microbial surface component recognizing adhesive matrix molecule adhesion of collagen from Enterococci), serine protease, capsule, cell wall polysaccharide and superoxide. [4-10]

This study was carried out to study the elaboration of virulence factors by strains of Enterococci isolated from clinical specimens and to determine if there is any significant difference in the virulence

factors found in clinical isolates and those in isolates from stool samples.

MATERIALS AND METHODS

This study was conducted in the department of Microbiology SRM Medical college Hospital and Research centre, Kattankulathur, from April 2013 to March 2014.A total of 128 clinical isolates of *Enterococcus* species were included in this study. Routine bacteriological methods were followed for the isolation and identification of *Enterococcus* species. [11,12] Thirty isolates of Enterococcus species from stool samples were collected for comparing the virulence factors elaborated by strains isolated from clinical samples and those found as commensals in stool

Bile- esculin azide agar was used for the isolation of *Enterococcus* species from stool specimens. [13]

Haemolysin

Enterococcal isolates were plated onto Brain heart infusion agar supplemented with 5% human blood. Plates were incubated at 37°C and observed after 24 and 48 hours. A clear zone of β- haemolysis around the colonies indicted the production of haemolysin [13]

Gelatinase

Peptone yeast extract agar containing gelatin (30g/L) was prepared and incubated at 37°C for 24 hours after inoculating *enterococcus* isolates .The plates with growth were then cooled to ambient temperature for 2 hours. Appearance of turbid halo was considered to be indicative of gelatinase production. [13]

Biofilm production

Qualitative and Quantitative detection of biofilm (adherence) was detected using microtitre plate method.

Microtitre plate method (MTP)

Colonies of Enterococci after overnight growth on blood agar were inoculated in Trypticase soy broth (TSB) and incubated at 37° C for 24 hours. The broth culture was diluted 1:10 with freshly prepared trypticase soy broth. 20 µl of broth culture was added to flat bottomed microtitre plate containing 230µl of TSB. A 96 well microtitre plate with flat bottom was used. First three wells served as media controls without the addition of cultures. 3 known in house positive and negative controls were inoculated in each plate. The test organism diluted in trypticase soy broth was inoculated in triplicate and incubated overnight at 37°C aerobically. After 24 hours of incubation, the plates were gently washed with PBS (pH 7.2) thrice, to remove the free floating planktonic bacteria. 300µl of methanol was added to each well and allowed to stand for 15 minutes. The excess of methanol was discarded; wells were stained using 0.1% Safranin stain. After 20 minutes of staining, excess stain was discarded and washed with phosphate buffer saline. The stained cells adherent to wells is resolubilised with 33% glacial acetic acid. Test was carried out in triplicate and average of three optical density values was taken. The optical density of each well was measured at 490nm using automated Biorad Laboratory reader. The OD readings were considered as an index of bacteria adhering to surface and forming bio films.

Biofilm formation was considered to be high when the absorbance was > 1.256 OD, moderate when it was between 0.6-1.2 and weak when between 0.3-0.6 and non-adherent/absent when <0.3. [14,15]

Comparison of the virulence factors elaborated by clinical isolates and isolates from stool

Thirty strains of Enterococcus isolated from stool samples were tested by the above - mentioned methods for the detection of hemolysin, gelatinase and bio film production.

Comparison of the virulence factors expressed by clinical isolates and isolates from stool were done using Chi-square test.

RESULTS

One hundred and twenty eight clinical isolates and thirty Enterococcus isolates from stool were tested for the presence of virulence factors.

Virulence factors in *Enterococcus* isolates from clinical samples

Hemolysin production in Enterococci

Of the 128 samples tested for the presence of hemolysin, 36.8% showed beta-hemolysis on human blood agar.45.8% of the urine isolates showed beta-hemolysis whereas only 26.2% of blood and exudates tested positive for hemolysin.

Gelatinase production in Enterococci

Gelatinase production was seen in only 23.5% of clinical isolates 24.3% of urine isolates were positive for gelatinase activity in contrast to 31% from blood and exudates.

Biofilm Production by isolates Of Enterococci detected by microtitre plate method (MTP)

Among the clinical isolates tested for biofilm production, 55.5% were positive by microtitre plate assay.62.4% of enterococcal isolates from urine were found to be positive for biofilm formation by microtitre plate assay.

Table: 1 Virulence factors in clinical isolates of Enterococci

Virulence factor	No of clinical isolates tested	No of positive clinical isolates (%)
Hemolysin	128	47(36.8)
Gelatinase	128	25(19.6)
Biofilm	128	71(55.5)

Elaboration of virulence factors by Enterococcus isolates from stool

A total of thirty *Enterococcus* species isolates from stool samples were tested for the presence of virulence factors like hemolysin, gelatinase and biofilm production.

Table: 2 Virulence factors in Enterococcus isolates from stool samples

Virulence factor	No of stool isolates tested	No of positive stool isolates (%)
Hemolysin	30	3 (10)
Gelatinase	30	2 (6.7)
Biofilm production	30	8 (26.6)

Comparison between virulence factors expressed by *Enterococcus* isolates from clinical samples and those from stool samples.

Table: 3 Hemolysin productions in clinical and commensal isolates

	Hemolysin	
	Positive	**Negative**
Clinical isolates	47	81
Commensals	3	27
	Pvalue= 0.0047	Significant at P value < 0.05

Table: 4 Gelatinase productions in clinical and commensal isolates of Enterococci

	Gelatinase	
	Positive	**Negative**
Clinical isolates	30	98
Commensals	2	28
	P value = 0.004	Significant at P value <0.05

Table: 5 Biofilm productions in clinical and commensal isolates of enterococci

	Biofilms	
	Positive	**Negative**
Clinical isolates	71	57
Commensals	8	22
	P value =0.0045	Significant at P value <0.05

DISCUSSION

Enterococci have emerged as one of the leading pathogens in causing nosocomial infections and super infection in patients receiving antibiotics. Prompt identification of virulence factors associated with invasiveness and disease severity has become an inevitable subject for research.

In our study, expression of three potential virulence factors among clinical and commensal isolates were evaluated-hemolysin, gelatinase and biofilm formation. Our study shows that with respect to all the three virulence factors produced, there was significant difference between the clinical and commensal isolated ($p<0.05$).

Hemolysin production and gelatinase production was seen in 36.8% and 19.6 % of all clinical isolates tested when compared to 10% and 6.7% seen in commensal strains. Biofilm production was seen in 55.5 % of clinical isolates as to 26.6% seen in commensal strains. However, in few studies there has been no appreciable difference in significance of virulence factors produced by clinical and commensal isolates. [16]

Cytolysins (hemolysins) have been shown to be associated with increased

mortality in animal (rabbit) models of enterococcal endocarditis. Generally, the capability to produce the cytolysin (hemolysin) is plasmid mediated although occasionally, cytolysin genes can occur as chromosomal elements. [17] Cytolytic enterococci have been shown to be associated with bacteriocin production with activity against some gram-positive but not gram-negative bacteria. [18]

Gelatinase elaborated by some Enterococcus isolates has been identified to be an extracellular zinc-endopeptidase capable of hydrolyzing gelatin, collagen, casein, hemoglobin and other peptides. The role of gelatinase in causing endocarditis has been studied using animal models. [19] The role of gelatinase in enterococcal infection is in providing nutrition to the bacteria by degradation of host tissues.

Enterococci have been associated with biofilms on various kinds of indwelling devices like prosthetic heart valves, urinary catheters, artificial hip prostheses etc., and this capability to produce biofilms has been considered an important virulence factor of these organisms. Various methods like microscopic biofilm formation assay and epifluorescence microscopy have been tried to study the biofilm-forming capability of bacteria. However, the method that has been used very frequently in recent times is the microtiter plate biofilm production assay. This method is preferred because of its simplicity and cost-effectiveness. The microtiter plate biofilm assay technique was first devised for studying the biofilm-forming capability of Listeria monocytogenes by Djordjevic et al. This method was later modified and used for studying biofilm formation in other gram-positive bacteria like coagulase-negative staphylococci and Enterococcus species. [20]

Adherence to body surfaces is considered as the major factor responsible for the pathogenicity of clinical isolates of *Enterococcus*. Strains causing infections have higher propensity to adhere to surfaces than commensal strains. Development of certain measures like inhibition of the action of virulence factors or blocking the biofilm production may provide an alternate method of therapy in view of antimicrobial or multidrug resistance.

CONCLUSION

Significant difference in the production of virulence factors was noted between clinical and commensal isolates. The virulence factors were more prevalent in clinical isolates thereby causing infections and drug resistance.

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