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Research Article

PREVALENCE OF VIRULENCE FACTORS AMONG CLINICAL ISOLATES OF *ENTEROCOCCUS* SPP.

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ABSTRACT

Objective: To identify some of the virulence factors such as hemolysin, gelatinase, and biofilm production among the clinical isolates of enterococci.

Methods: Hemolysin detection using sheep blood agar. Gelatine agar was used for gelatinase production, and tube adherence method was used for detecting biofilm production.

Results: Hemolysin production observed in 49% of isolates, gelatinase production in 41% of isolates, and 46% of isolates were produced biofilm.

Conclusion: Virulence factors production was noticed more in *Enterococcus faecalis* than *Enterococcus faecium*. It is necessary to find the production of important virulence factors among the clinical isolates as they are always associated with virulence of the organism including drug resistance.

Keywords: Hemolysin, Gelatinase, Biofilm, *Enterococcus*.

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INTRODUCTION

Enterococci are Gram-positive, facultative anaerobic bacteria classified as Group D streptococci. They have developed well-adapted mechanism to survive in the gastrointestinal tract of the human being as a commensal, but these opportunistic bacteria sometimes become pathogen and cause dangerous infections such as bacteremia, endocarditis, urinary tract infections, intra-abdominal and pelvic infection, surgical site infection, and diabetic foot infection [1].

Among the *Enterococcal* isolates, *Enterococcus faecalis* and *Enterococcus faecium* are the leading cause of nosocomial infections (*E. faecalis* 85-90% and *E. faecium* 5-10%). Several potential virulence factors have been identified in enterococci, but none has been established as having a major contribution to virulence in human [2].

Hemolysin also called as cytolysin and is a post-translationally modified protein toxin that occurs in many strains of enterococci [3]. In *E. faecalis,* it can occur up to 60%. Cytolysin causes rupture of different target membranes, including erythrocytes, bacterial cells, and other mammalian cells [4]. Hemolysin played a role in human infections was proved in Japan; about 60% clinical isolates from *Enterococcal* infections were hemolytic compared with only 17% of isolates from feces of healthy individuals [5]. Gelatinase (Gel E) is an extracellular protease which is produced by enterococci and is consider as one of the important virulent factors. It is capable of hydrolyzing gelatine, casein, collagen, hemoglobin, and other peptides [4]. Gelatinase of *E. faecalis* gene is cotranscribed with sprE gene encoding serine protease. These two proteases give nutrition to bacteria and directly or indirectly cause damage to host tissues [6]. Proteolytic activity of gelatinase contributes an important role in the pathogenicity of *E. faecalis* [7].

Enterococci has an ability to produce biofilm on abiotic surfaces is said to be another important virulence factor [8]. The enterococci have been associated with biofilm in endocarditis, root canal infection, and ocular infection and urinary tract infection and in a variety of device-related infection in which biofilm was found on intrauterine devices, prosthetic heart valves, artificial hip prostheses catheters, and stents [9]. Biofilm production is nosocomial strains of organisms is an important pathogenic factor in causing infection in the hospital environment [10].

Our study was aimed to identify different species of enterococci isolated from the clinical samples and also to identify some of the virulence factors such as hemolysin, gelatinase, and biofilm production among the clinical isolates of enterococci.

METHODS

Clinical isolates of *Enterococcus* were identified phenotypically by standard biochemical tests up to species level.

Species identification

Species identification was done by motility testing, pigment production, and fermentation from various sugars and pyruvate [2,11].

Motility

Motility was observed by Hanging drop preparations. All enterococci are non-motile except *Enterococcus gallinarum, Enterococcus casseliflavus,* and *Enterococcus flavascens,* which are not common human pathogens.

Pigment production

Pigment production is easily detected in nutrient agar plates. Inoculate the organism in the nutrient agar plate and incubate at 37°C for overnight and observed for any pigment production. Common pathogenic enterococci are not producing pigment. Some enterococci are pigment producing such as *Enterococcus mundtii, E. casseliflavus, E. flavascens,* and *Enterococcus sulfureus.*

Sugar fermentation

Mannitol fermentation, arabinose fermentation, raffinose fermentation, and pyruvate fermentation were done to speciate *Enterococcus*

Detection of virulence factors

Hemolysin (cytolysin) production

Hemolysin production was detected by inoculating enterococci onto freshly prepared blood agar plates. Plates were incubated at 37°C and evaluated after 24 and 48 hrs. A clear zone of β-hemolysis around the stab or streak on human blood agar was considered to be a positive indication of hemolysin production [2,12].

Production (Fig. 1)

Gelatine agar is prepared by adding gelatine to nutrient medium. The organism is inoculated in gelatine agar plate and incubated at 37°C for 24 hrs. Then, the plate is flooded with mercuric chloride and clear zone around the colonies indicate gelatinase production [2,12].

Biofilm production

Tube adherence method (Fig. 2)

Trypticase soy broth 10 ml was taken in sterile test tubes and was inoculated with loopful of micro-organism from overnight culture plates and incubated for 24 hrs at 37°C. The tubes were decanted and washed with phosphate buffer solution (P_H 7.3) and dried. The dried test tubes were stained with crystal violet (0.1%). Excess stain was removed, and tubes were washed with deionized water. Tubes were then dried in inverted position and observed for biofilm formation. The biofilm formation was positive when a visible film lined the wall and bottom of the tube.

Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined, and amount of biofilm formation was scored as 0-absent, 1-week, 2-moderate, and 3-strong [2,12].

RESULTS

Out of 100 isolates of *Enterococcal* spp. from various clinical specimen received from our hospital, we identified 89 (89%) *E. faecalis* and 11 (11%) *E. faecium*.

Out of 89 isolates *of E. faecalis* identified, 72 (83%) from urine samples, 14 (15.7%) from pus samples, and 3 (3.3%) from blood samples. Out of 11 isolates of *E. faecium* identified, 8 (72.7%) from urine samples and 3 (27.2%) from pus samples (Table 1).

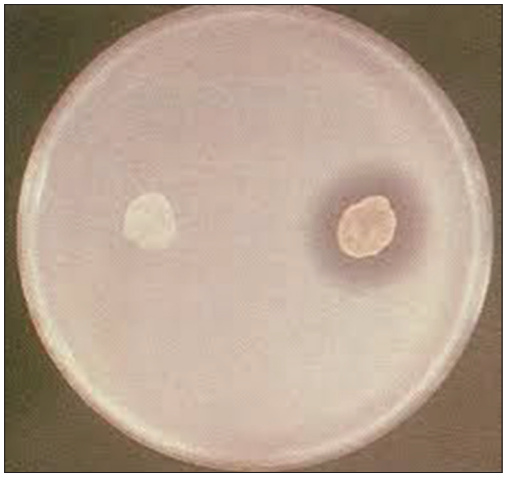

Fig. 1: Gelatinase production

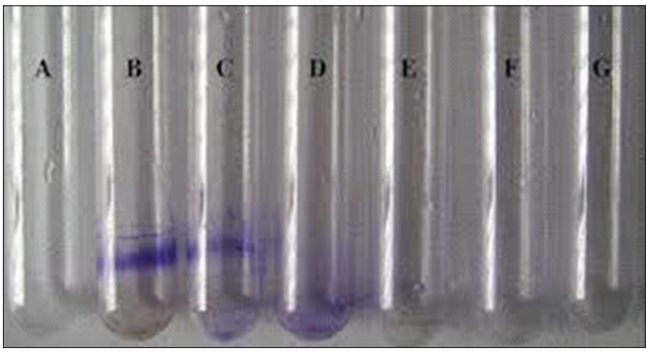

Fig. 2: Tube adherence test for biofilm production

All isolates were detected for virulence factors production. Common virulence factors in *Enterococcal* spp. are hemolysin, gelatinase, and biofilm. Among 89 E. *faecalis,* 46 (51.68%) were producing hemolysin, 39 (43.8%) were producing gelatinase, and 42 (47.19%) were producing biofilm. Out of 11 *E. faecium*, 3 (27.27%) producing hemolysin, 2 (18.18%) were producing gelatinase, and 4 (36.36%) were producing biofilm. A total of 49 (49%) hemolysin, 41 (41%) gelatinase and 46 (46%) biofilm were produced (Table 2).

Some of the isolates showed production of more than one virulent factors. 22 (21%) isolates were identified as multiple virulent factors producers. 11 (11%) isolates were producing all three virulent factors included in our study. 10 (10%) isolates were produced two virulent factors. Of which, 6 of them produced both biofilm and hemolysin, 3 isolates were produced both biofilm and gelatinase, and only 1 showed the production of hemolysin and gelatinase.

DISCUSSION

Enterococci once considered a harmless commensal survive in the gastrointestinal tract of human has emerged as a medically important multidrug-resistant virulent pathogen causing outbreaks of many dangerous nosocomial infections such as bacteremia, endocarditis, urinary tract infections, intra-abdominal and pelvic infection, surgical site infection, and diabetic foot infection [1].

In our study, 100 strains of *Enterococcal* spp. were isolated from various clinical samples during a period of 1-year. Among 100 isolates, 89 (89%) were identified as *E. faecalis*, and 11 (11%) were identified as *E. faecium*. In 2008, a study by Jayanthi *et al*. reported that 80-90% of all *Enterococcal* infections were caused by *E. faecalis* [13]. A study from London, Teixeira *et al*. reported that *E. faecalis* and *E. faecium* are the leading cause of nosocomial infection (*E. faecalis* 85-90% and *E. faecium* 5-10%) [2]. In the year 2005, a study by Coque *et al*. showed isolation of 77% *E. faecalis* and 15% *E. faecium* from blood [14]. *E. faecalis* is still being the most common species among *Enterococcus* isolated from clinical specimens.

All isolates were tested for virulence factors production. The presence of virulence factors indicates the severity of pathogenicity in any bacteria. Common virulence factors in *Enterococcal* spp. such as hemolysin, gelatinase, and biofilm were studied. In our study, 46 (51.68%) *E. faecalis* strains and 3 (27.27%) *E. faecium* were produced hemolysin. Hemolysin also called as cytolysin and is a modified protein toxin that occurs in many strains of enterococci [15]. A study from Bangalore showed that 16.5% clinical and 19% commensal isolates produce hemolysin which is less when compared to our study. Another study from Turkey by Gulhan *et al*. reported that hemolysin production was detected in 50% of *E. faecalis* isolates and 18.2% in *E. faecium*

Table 1: *Enterococcus* species isolated from various samples

Samples	*E. faecalis* (n=89)	*E. faecium* (n=11)	Total (n=100)
Urine (%)	72 (83)	8 (72.7)	80 (80)
Pus (%)	14 (15.7)	3 (27.2)	17 (17)
Blood (%)	3 (3.3)	0 (0)	3 (3)

E. faecalis: Enterococcus faecalis, E. faecium: Enterococcus faecium

Table 2: Prevalence of virulence factors in *Enterococcus* isolates

Virulence factors	*E. faecalis* n=89	*E. faecium* n=11	Total n=100
Hemolysin (%)	46 (51.68)	3 (27.27)	49 (49)
Gelatinase (%)	39 (43.8)	2 (18.18)	41 (41)
Biofilm (%)	42 (47.19)	4 (36.36)	46 (46)

E. faecalis: Enterococcus faecalis, E. faecium: Enterococcus faecium

isolates [16]. In 2012, a study conducted by Dahlen *et al*. reported that hemolysin was detected in 16.7% of the *E. faecalis* strains, and none of the *E. faecium* isolates produces hemolysin [15]. In the year 2008, Jankoska *et al*. reported that occurrence of hemolysin up to 60% of clinical isolates and also reported that 50% of *E. faecalis* produced hemolysin [4]. In our study, we found that hemolysin was mainly produced by *E. faecalis* species than *E. faecium* which was reported by most of the other studies also. Most of the studies reported less rate of hemolysin production by both the isolates. Hemolysin played a role in human infections was proved in Japan; about 60% clinical isolates from *Enterococcal* infections were hemolytic compared with only 17% of isolates from feces of healthy individuals [5]. However, in our study, as we have not compared clinical isolates with commensals from healthy individuals, that cannot be proved which is one of the drawbacks of our study.

Gelatinase is an extracellular protease and is considered as one of the important virulent factors produced by enterococci. Gelatinase has a proteolytic activity which contributes an important role in the pathogenicity of *E. faecalis* [6]. In our study, 39 (43.8%) isolates of *E. faecalis* and 2 (18.18%) isolates of *E. faecium* were producing gelatinase. A study from India by Upadhyaya *et al*., in the year 2009, reported that 39% clinical isolates were producing gelatinase [10]. Another study by Strzelecki *et al*. from Poland reported that, of 153 *E. faecalis*, 53% of strains producing gelatinase, and among these, isolates from CSF were commonly producing gelatinase (75%) followed by isolates from urine (71%) less frequently by isolates from wound and blood. In the year 2000, a study by Qin *et al*. reported that 62% of *E. faecalis* produced gelatinase and the presence of gelatinase contributes to the virulence of *E. faecalis* [17]. In the year 2008, Jankoska *et al*. reported that the occurrence of gelatinase production was identified in 68% of *E. faecalis* of urine sample [4]. A study by Gulhan *et al*. found gelatinase production was common in both *E. faecalis* and *E. faecium* [16]. In our study, we found that gelatinase was largely produced by *E. faecalis* than *E. faecium*, and this was proved by most of the other studies.

Enterococci has an ability to produce biofilm on abiotic surfaces is said to be an important virulence factor [18]. Biofilm is an assembly of microbial cells associated with a surface and enclosed in a matrix of primarily polysaccharide material. The biofilm producing enterococci have been associated with endocarditis, root canal infection, and ocular infection and urinary tract infections and in a variety of device-related infections such as intrauterine devices, prosthetic heart valves, artificial hip prostheses catheters, and stents [8]. In our study, 42 (47.19%) isolates of *E. faecalis* and 4 (36.36%) isolates of *E. faecium* were producing biofilm, respectively. Upadhyaya *et al*. from India reported that 32% of clinical isolates were producing biofilm, but only 16% of commensal isolates were producing biofilm [10]. The worldwide biofilm producing prevalence was varied from each other country. In the United States, a study by Mohamed *et al*. reported that 93% of *E. faecalis* identified as biofilm producing isolates [19]. In the year 2008, Jankoska *et al*. reported that the occurrence of biofilm was found in 76% of *E. faecalis* from the urine isolates [4]. The production of biofilm is greater in gelatinase producing isolates than non-gelatinase producing isolates [17].

Two or more virulence factors present in some strains of *E. faecalis*, whereas 72.1% of *E. faecalis* were present with any one of the virulence factors most frequently biofilm production [20]. In our study also, there were isolates which produced multiple virulent factors. A total of 21% of isolates have shown production of all three virulent factors which we have studied. There was no clinical significance proved due to their multiple virulent factor production. Most of the foreign studies showed that more than 75% of *E. faecalis* produced Biofilm, but in our study, we found only 47% of *E. faecalis* were producing biofilm. Another Indian study showed that the low prevalence of biofilm production. Our study correlates with the Indian study proved that biofilm production was low in India. Biofilm production has got more significance especially if the isolate is from any medical device. A study from Iran in 2015 proved an association of biofilm production with drug resistance [21]. This has to be taken into consideration for selecting empirical antibiotic before culture report. Because near about 50% of isolates in our study showed biofilm production which reflects drug resistance percentage also.

CONCLUSION

The prevalence of Virulence factors, which we have been studied, is high in *E. faecalis* than *E. faecium*. As these virulence factors' production is associated with drug resistance in that particular isolate, these type of studies will give an idea about the prevalence of such virulence factors which will help in the treatment of the patients infected with *Enterococcus* species. Our limitation in this study is that we have not compared pathogenic strains isolated from the clinical specimen with the commensals from healthy individuals.

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