Original Article

Comparative study among clinical and commensal isolates of *Enterococcus faecalis* for presence of *esp* gene and biofilm production

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Abstract

Introduction: Because of increasing difficulty in treating enterococcal infections, effort is being devoted to understanding factors that are responsible for causing nosocomial infection, with a focus toward targeting these factors with new therapeutics. Evidence has emerged that the *esp* gene mediates biofilm formation *in vitro,* which helps the organism colonize and cause infection.

Methodology: This study was conducted over a four-year period in a tertiary-care hospital. There were 200 clinical pathogenic strains isolated from nosocomial infections and 100 commensals from stool specimens of healthy individuals. The study compared the production of biofilm and detection of the *esp* gene among clinical and commensal isolates.

Results: Among 200 clinical isolates of *Enterococcus faecalis* 65 (32.5%) isolates were positive for biofilm production and 60 (30%) for the *esp* gene by PCR. Among 100 commensal isolates, 16 (8%) and 14 (7%) were positive for biofilm formation and the *esp* gene, respectively. Five clinical and two commensal isolates produced biofilm without any amplification of the *esp* gene.

Conclusion: The study shows a significant difference in production of biofilm and presence of the *esp* gene between clinical and commensal isolates ($P < 0.002$). Therefore, it can be concluded that biofilm production has an important role in causing nosocomial infection. Although detection of the *esp* gene correlates with biofilm production, it may not be the only factor determining the formation of biofilm since few isolates produced biofilm without the *esp* gene. Strains isolated from indwelling medical devices showed high production of biofilm and *esp* gene.

Key words: *Enterococcus*; nosocomial; virulence; biofilm; *esp* gene

J Infect Dev Ctries 2011; 5(5):365-369.

(Received:12 July 2010 – Accepted: 05 November 2010)

Introduction

Enterococci are commensal organisms of gastrointestinal (GI) tracts. While more than 100 years ago they were recognized as being capable of causing serious infection in humans [1], over the past three decades enterococcal strains have emerged as the leading organisms causing nosocomial infections. They are resistant to virtually all antibiotics, including vancomycin and linezolid, the last resort of treatment for Gram-positive infections. Enterococci occur in the colon of nearly all humans, where they can be found in numbers as high as 10^8 colony-forming units (CFU) per gram of faeces [2,3]. This is evidence that enterococci have successfully evolved traits that enable stable colonization of this highly competitive niche. *Enterococcus faecalis* currently accounts for 65-80% of all enterococcal nosocomial infections [2,4].

It is well-known that virulence of an organism is regulated by virulence coding genes present in special regions of the genome which are termed pathogenicity islands (PAI). The enterococcal PAI was first identified in the genome of a multidrug-resistant strain of *E. faecalis* [MMH594] that had caused an outbreak of nosocomial infection in the 1980s [5].

Direct experimental evidence suggests that the Esp protein acts as an adhesin in the colonization of the urinary tract [6]. Evidence has emerged that the *esp* gene mediates biofilm formation *in vitro* [7]. Shankar *et al*. (2001) found that 93.5% of *esp* gene-harboring *E. faecalis* strains formed biofilm on polystyrene, whereas none of the *esp*-deficient strains did [06]. The *esp* gene has been detected in close association with the cytolysin operon on the chromosome of the *E. faecalis* ward outbreak strain

Table 1. Demographic and clinical characteristics of 200 patients with nosocomial infection due to *E. faecalis*

Characteristics	Number of cases (%)
Age, years (range)	16-66
Male / female	131/69
Post operative wound infection	125 (62.5)
Endotracheal tube infection	23 (11.5)
Diabetic foot infection	20(10)
Urinary tract infection	15 (7.5)
Infected compound fracture	7 (3.5)
Septicemia	5 (2.5)
Burn cases	3 (1.5)
Umbilical catheter tip infection	2 (1)

MMH594 [8]. The cytolysin operon and the *esp* gene are found within a region that appears to be subjected to rearrangement. The association of adhesion with toxins has been recognized among isolates of *E. coli* from urinary tract infections [9-11]. It can be hypothesized that on the basis of the proximity of the *esp* gene to the cytolysin operon within this region and its role in generating microenvironments where a quorum of bacteria would accumulate (*e.g.*, biofilms), the *esp* gene may function synergistically with the cytolysin in a manner analogous to the previously documented synergy between aggregation substance and cytolysin in endocarditis.

Methodology

This study was conducted from July 2004 to July 2009. Three-hundred and ninetyseven *Enterococcus* spp were isolated from different clinical nosocomial samples. Two hundred isolates were confirmed as *E. faecalis* by biochemical reactions [12]. The demographic distribution of the clinical isolates is mentioned in Table 1. One hundred commensal isolates of *E. faecalis* were isolated from stool samples.

For investigation of biofilm production, bacterial strains were grown in tripticase-soy broth (TSB, from Hi-Media, Mumbai, India) with 0.5% glucose and incubated at 37°C [13]. After overnight incubation, culture was diluted 1:40 in fresh TSB-0.5% glucose. Two hundred microlitres of the diluted solution was added to wells of a flat-bottomed polystyrene microtitre plate and incubated for 48 hours at 37ºC. Wells were gently washed with distilled water and the plate inverted and dried at room temperature for one hour. The adherent biofilm was stained with 0.1% saffranin dye and incubated 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 ELISA reader (Tecan, Männedorf, Switzerland). The test was conducted in triplicate for concurrence. Biofilm producing *esp* gene negative *E. faecalis* OG1RF was taken as the positive control. The mean optical density (OD) value of the positive control was taken as the reference value to be compared against. Values above 0.2 were considered as high biofilm producers; values below 0.081 were categorized into low or non-biofilm producers. OD values above the reference value but within 0.081 and 0.2 were taken as moderate biofilm producers.

All the clinical and commensal isolates were tested for the presence of the *esp* gene by PCR . DNA extraction was performed by phenol-chloroform extraction. PCR amplification of the *esp* gene was performed using primers *esp* 11 (5′-TTGCTAATGCTAGTCCACGACC-3′) and *esp* 12 (5′-GCGTCAACACTTGCATTGCCGAA-3′), corresponding to nucleotide positions 1217-1238 and 2149-2171, respectively, within the N-terminal region of the *esp* gene [14]. The PCR reaction mixture consisted of 250 ng of DNA sample; 0.2 µL each of dATP (2′-deoxyadenosine 5′-triphosphate), dCTP (2′-deoxycytosine 5′-triphosphate), dGTP (2′-deoxyguanosine 5′-triphosphate), and dTTP (2′-deoxythymidine 5′-triphosphate); 2.5 mM $MgCl_2$; and 2.5 U of AmpliTaq DNA polymerase in 1X reaction buffer (Bangalore Genei, Bangalore, India). The samples underwent initial denaturation at 95°C for two minutes then were subjected to 40 cycles of denaturation (94°C for 45 seconds), annealing (63°C for 45 seconds) and extension (72°C for 1 minute). The amplification mixture was subjected to electrophoresis. The reaction products were visualized by ethidium bromide staining. Reference isolates of *E. faecalis* MMH594 (which carried the *esp* gene) and OG1RF (which did not carry the *esp* gene) were used as positive and negative controls, respectively. The distributions of these two virulence factors among the different clinical specimens were also verified.

Statistical analysis

Statistical analysis of the data was produced with SPSS software (IBM, Rochester, NY version 15). Chi-square test and *P* values were calculated, and a significant difference was noted among clinical and commensal isolates with respect to production of biofilm and *esp* production.

Table 2. High, moderate and non-biofilm producers among clinical and commensal isolates

Biofilm formation $OD_{490\ nm}$	Clinical isolates (n = 200)	Commersal isolates (n = 100)
High (> 0.20)	23 (11.5%)	4 (4.0%)
Moderate(>0.081 and < 0.20)	42 (21.0%)	12 (12.0%)
Weak/non-biofilm producers (< 0.081)	135 (67.5%)	84 (84.0%)

Chi-square test. (X-squared = 9.653, df = 2, *P* value = 0.008015)

Table 3. Comparison among clinical and commensal isolates for production of biofilm and *esp* gene (*P* value > 0.05)

Factor	Clinical isolates (n = 200)	Commensal isolates (n = 100)
Biofilm	65 (35.0%)	16 (8.0%)
Esp gene	60 (30.0%)	14 (7.0%)

Results

Production of biofilm among clinical and commensal isolates

Out of the 200 clinical isolates, 23 samples showed an OD value greater than 0.2, suggesting high biofilm producing strains; 42 samples showed an OD greater than the positive control but below 0.2, suggesting moderate biofilm-producing strains. Of the 100 commensal isolates, only four samples showed high production of biofilm, and 12 samples showed moderate production of biofilm as shown in Table 2.

Presence of esp gene among clinical and commensal isolates

Analysis for the presence of the *esp* gene in clinical isolates and commensals found 60 (30%) positive and 14 (14%) positive, respectively. Statistical analysis provided an X-squared value of 9.1844 and a significant *P* value (*P* = 0.002441)

Further correlation between biofilm formation and presence of the *esp* gene was observed in the majority of clinical isolates as shown in Table 3, however a comparative study of biofilm production and presence of the *esp* gene showed that there were five clinical isolates and two commensal isolates that produced biofilm but were negative for *esp* gene**.**

Further analysis of clinical isolates revealed that 21.6% and 19.2% of strains isolated from post-operative wounds contained biofilm-formers and were positive for *esp* gene, respectively. In samples from cases of endotracheal tube infection, urinary tract infection, and umbilical catheter tip infection, 60.9%, 86.7% and 100% isolates showed biofilm production and 56.2%, 80.0% and 100% were found positive for the *esp* gene, respectively, as shown in Table 4.

Discussion

This study shows a significant presence of biofilm formation and the *esp* gene in nosocomial infections. It is evident from the study that biofilm formation is important in bacterial pathogenesis. Biofilms play a significant role in colonization during infection, providing an opportunity for the bacteria to develop drug resistance. Biofilm forming bacteria are encased in a well-hydrated matrix composed of secreted exopolymeric substances, proteins and nucleic acids from dead-lysed cells that affords protection against host immune clearance and antibiotic therapy [15]. Besides tissue epithelia, bacterial biofilms can also develop on body implant devices [16]. It is conceivable that the increasing use of antibiotics and implant devices in hospitals contribute to the enrichment of traits that promote biofilm development in clinical pathogens [16-18]. Similarly, *esp* gene presence showed a marked difference between clinical and commensal isolates. There were five clinical isolates and two commensal isolates that produced biofilm independent of the *esp* gene. Another study has also shown such strains [19]. There was no significant difference among the clinical and commensal isolates with respect to absence of the *esp* gene (P > 0.05). The results

Table 4. Comparison between biofilm production and *esp* gene among different clinical specimens

Clinical samples	Biofilm formation (%)	Presence of esp gene (%)
Postoperative wound infection (n = 125)	27(21.6)	24 (19.2)
Endotracheal tube infection (n = 23)	14 (60.86)	13 (56.2)
Diabetic foot infection (n = 20)	5 (25.0)	5 (25.0)
Urinary tract infection (n = 15)	13 (86.66)	12 (80.0)
Infected compound fracture (n = 7)	2 (28.57)	2 (28.57)
Septicaemia (n = 5)	2 (40.0)	2 (40.0)
Burn cases (n = 3)	0	0
Umbilical catheter tip infection (n = 2)	2 (100)	2 (100)

indicate that there may be more than one factor determining the production of biofilms in enterococci. Biofilm formation may be an adaptive response of the organism; therefore, other virulence factors also need to be explored.

Based on the distribution of biofilm production and presence of the *esp* gene in various clinical specimens, it is observed that the *esp* gene and biofilm formation are more prevalent in the organisms isolated from endotracheal tubes (60.8%), Foley's catheter tips (86.6%) and umbilical catheter tips (100%). On that basis, we hypothesize that biofilm and the *esp* gene are important factors responsible for the ability of *E. faecalis* to colonize and cause nosocomial infections associated with indwelling medical devices. Moreover, strains that did not form biofilm could be converted to a biofilm-former phenotype by transformation to an *esp* positive strain. An Esp-like protein that occurs in staphylococci was shown to mediate biofilm formation, indicating that this trait is wide-spread among organisms that cause nosocomial infections related to indwelling devices [20-24].

Use of indwelling medical devices such as catheters, mechanical heart valves and prosthetic joints is associated with an increased risk of infection within the blood-stream and urinary tract. Bacteria also exhibit slower growth rates and greater tolerance to antimicrobial agents when growing in a biofilm [21]. The process of biofilm formation is complex and dependent upon multiple factors including properties of the substratum, presence of a conditioning film, hydrodynamics, physical and chemical properties of the liquid in contact with the device surface, and properties of the colonizing microbial cells [22]. Biofilm-associated bacteria may elicit disease processes by detachment of individual cells or aggregates from the device surface, production of endotoxins or providing a niche for the development of antibiotic-resistant organisms [21]. Biofilm-associated organisms also exhibit tolerance to antimicrobial agents [23]. This tolerance makes the treatment of device-associated infections with systemic antimicrobial agents generally ineffective.

A variant of the *esp* gene was detected in all epidemic vancomycin-resistant *E. faecium* (VRE) in hospitals but not in non-epidemic animal isolates [24]. This observation indicates that the surface protein Esp is associated with enterococcal colonization and spread. Analysis of the mechanism underlying the influence of this surface protein on transmission of enterococci might lead to new ways to prevent colonization and transmission.

As drug resistance is a major problem in enterococci, it is important to prevent the colonization of the organism by finding novel methods to prevent biofilm formation. Currently, there is more urgency to define the reservoirs for colonization and the routes of transmission of enterococci since few therapeutic options exist for treatment of VRE infections.

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Conflict of interests: No conflict of interests is declared.