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Spread of Enterococcal Surface Protein in Antibiotic Resistant *Enterococcus faecium* and *Enterococcus faecalis isolates* from Urinary Tract Infections

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Abstract: Enterococci rank among leading cause of nosocomial bacteremia and urinary tract infection in hospital and community acquired infections. Several traits that may contribute to enhanced virulence have been identified in Enterococci. Extracellular surface protein (Esp) is a virulence factor that contributes in biofilm formation and resistance to environmental stresses. In this study we aimed to determine occurrence of *esp* in *E. faecium* and *E. faecalis* isolates isolated from urinary tract infections and to investigate whether there is any correlation between presence of *esp* and antibiotic resistance. One hundred and sixty six isolates were collected from patients with UTI and after identification by biochemical and PCR, antibiotic resistances were examined. The presence of *esp* was investigated by primer-specific PCR. 43.3% of isolates identified as *E. faecium* and 56.7% as *E. faecalis*. The *esp* gene was found in 76.1% of *E. faecium* isolates and 77.9% of *E. faecalis* isolate. There were significant correlation between *esp* positive *E. faecium* and resistance to Vancomycin ($p<0.01$), also in *E.faecalis* we found correlation between *esp* positive and resistance to Ampicillin, Chloramphenicol and Tetracycline ($p<0.01$, $p<0.01$, $p<0.01$ respectively). Occurrence of *esp* in our isolates from urinary tract infection was high that indicates importance of this gene in urinary tract infections and shows importance of ability to forming biofilm and hydrophobicity of surface of Enterococci for causing urinary infection by Enterococci. Also, our finding showed significant correlation between resistance to antibiotics and presence of *esp* in Enterococci.

Keywords: Antibiotic resistance, *e. faecium*, *e. faecalis*, *esp*, urinary tract infection.

INTRODUCTION

For many years *Enterococci* species believed to be harmless and considered medically unimportant, because they produce bacteriocins, and they have been used in food industry as starter for dairy products and probiotic [1], but recently *Enterococci* have been emerged as an important nosocomial pathogens and are among the most frequently isolated organisms in hospital-acquired infections [2]. At present *Enterococci* is known as important nosocomial infections such as endocarditis [3] bacteremia [3, 4] and urinary tract infections [5]. The majority of enterococcal infections are caused by *E. faecalis*. However, in parallel with the increase in nosocomial *Enterococci* infections, a partial replacement of *E. faecalis* by *E. faecium* took place in the world [6, 7]. Little is known about enterococcal properties which make it possible for the bacteria to adhere and colonize the host tissue [8]. Several traits that may contribute to enhanced virulence have been identified in *Enterococci*, but better understanding of the virulence of *Enterococci* is necessary to control further spread and developing new treatment strategies [6].

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Extracellular surface protein (*esp*) is a cell wall associated protein first described in *Enterococcus* species by Shankar *et al.* [9]. It is thought to promote adhesion, colonization and evasion of the immune system, and to play some role in antibiotic resistance [1]. It has also contributions in biofilm formation of *Enterococci*, which lead to resistance to environmental stresses, and adhesion to eukaryotic cells, such as those of the urinary tract [10]. Shankar *et al*. showed contribution of *esp* in the persistence of *Enterococci* in the animal model of urinary tract infections [11]. In this study we aimed to determine presence of *esp* gene in *E. faecium* and *E. faecalis* isolated from urinary tract infections and to investigate any relationship between presence of *esp* gene and rate of antibiotic resistance.

MATERIALS AND METHODS

Study Design

One hundred and sixty six clinical isolates of *Enterococci* were collected from patients with urinary tract infections. The isolates were identified as *Enterococci* and specified by biochemical tests [12]. Identification was confirmed by Mass Spectrophotometer (MALDI-TOF MS microflex, 2012 bruker, Germany) and PCR (as described below). Antibiotic resistance properties of strains were examined by kerby-bauer method according to CLSI guideline (CLSI M100-S24) [13].

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DNA Extraction and PCR

DNA extraction was done by Cinnapure™ DNA extraction kit (Cinnagen, Iran). Briefly, bacterial pellet was resuspended in 100 µl gram positive prelysis buffer and added 20 µl lysosyme and incubated at 37°C for at least 30 min. After adding lysis buffer and precipitation solution, the solution was transferred to a spin column and after washing the spin, DNA was eluted by elution buffer in 65°C [14]. PCR was performed in 25 µl volumes that contained 20-200ng DNA, 0.5 µM of specific primers for *E. faecalis* ddlE1:ATCAAGTACAGTTAGTCTTTATTAG ddlE2: ACGATTCAAAGCTAACTGAATCAGT) [15], *E. faecium* (ddlF1: TTGAGGCAGACCAGATTGACG, ddlF2: TAT-GACAGCGACTCCGATTCC) [16] and for *esp* (espA: GGAACGCCTTGGTATGCTAAC, espB: GCCACTTTAT-CAGCCTGAACC) [9] with 1.5 mM MgCl2, 200 µM of each dNTP, 1X PCR buffer and 2 U DNA polymerase (Cinnagen, Iran). DNA was amplified by general PCR. An initial denaturation of 10 min at 94°C was followed by 35 cycles of denaturation at 94°C (1 min), annealing at 58°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. product length were 941bp for *E. faecalis*, 658bp for *E. faecium* and 95bp for *esp*. Positive control for PCR were *E. faecalis* MMH594 (also *esp* positive), *E. faecalis* 29212, *E. faecium* C38 and C68. Negative controls consisted of the PCR components on reaction mixtures lacking *Enterococci* DNA. PCR products were electrophoreses in 1.5% agarose gels and after staining with 0.5µg/ml ethidium bromide visualized under UV light. The size of fragments was determined in comparision with 100bp DNA ladder plus size marker (Fermentas., Germany).

Statistical Analysis

Chi-square test (or Fisher exact test) was performed for data analysis. *P* values below 0.05 were considered to be significant. Statistical analysis was done by Spss. 21 software.

Results

From 166 clinical isolates of urinary tract infections of *Enterococci*, 59.7% of isolates were from females and 40.3% from males. By biochemical differentiation and PCR for specific genes, 43.3% of isolates were identified as *E. faecium* and 56.7% as *E. faecalis*. Antibiotic resistance of strain described by disc diffusion (Figs. **1** and **2**) and the presence of *esp* gene was identified in 77.1% of all isolates of *Enterococci*. *esp* gene was identified in 76.1% of *E. faecium* and 77.9% of *E. faecalis* isolates (Fig. **3**). There was not any significant difference between presence of *esp* gene in *E. faecalis* and *E. faecium* isolates (P> 0.05). Correlation of resistance to antibiotic and presence of the *esp* genes was investigated. In *E. feacium*, correlation was found between presence of the *esp* and resistance to Vancomycin (p<0.01) (Fig. **1**). In *E. faecalis* we revealed correlation between resistance to Ampicillin, Chloramphenicol and Tetracycline (p<0.01, p<0.01 and p= 0.006 respectively) and presence of *esp* gene (Fig. **2**).

DISCUSSION

In the previous studies, several investigators have focused on the relationship between *Enterococci esp* gene and antibiotic resistance. The current investigation examined the relationship of *Enterococci esp* gene with antibiotic resistance of isolates from in the urinary tract infection. In this study we investigated the presence of *esp* in *E. faecium* and *E. faecalis* isolates from urinary tract infection. Enterococcal surface protein (*esp*) is considered as virulence factor in *Enterococci*, a few studies have done on presence and importance of *esp* on urinary tract infection of *Enterococci*. *esp* has been identified in *E. faecium* and *E.*

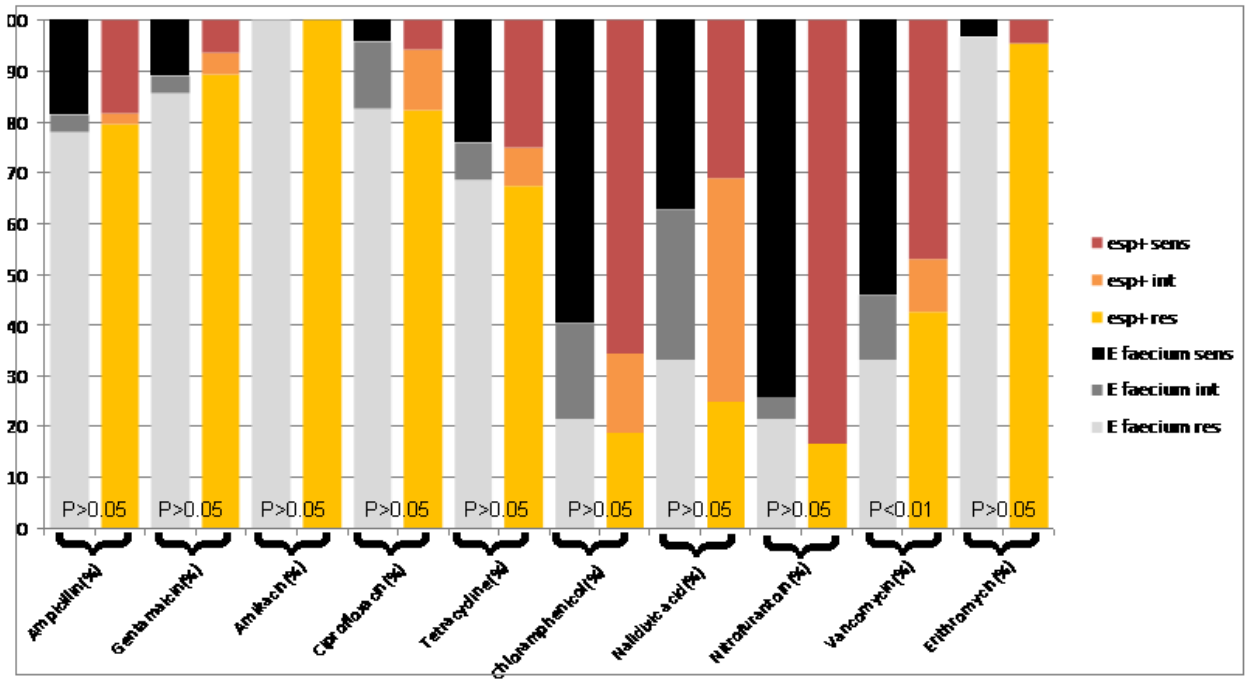

Fig. (1). *E. faecium* Antibiotic resistance properties in Total and Esp positive strains. Sens: Sensitive; int: intermediate; res: resistance.

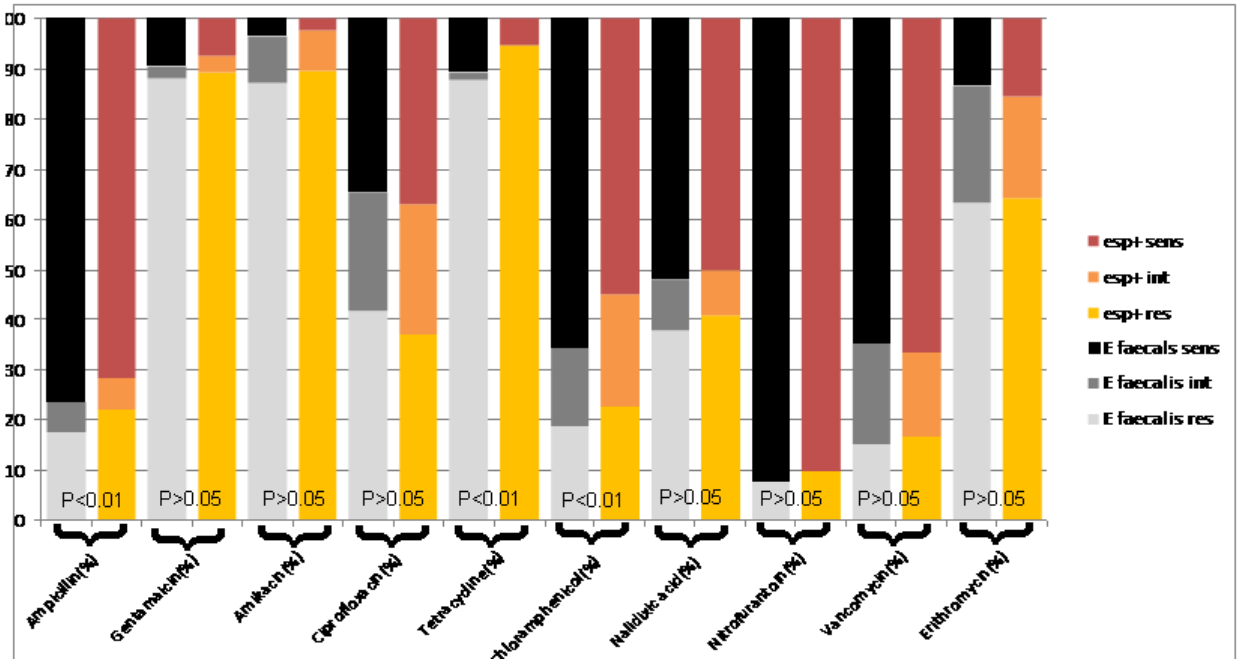

Fig. (2). *E.faecalis* Antibiotic resistance properties in Total and Esp positive strains. Sens: Sensitive; int: intermediate; res: resistance.

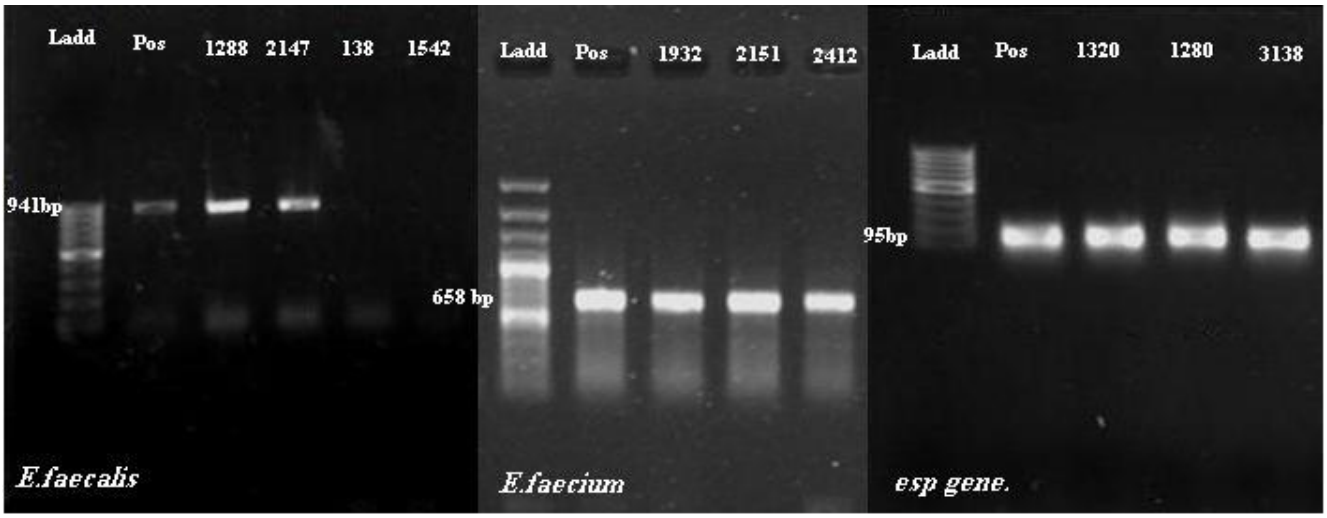

Fig. (3). PCR amplification and screening of Sample for presence of genome. Numbers intended in pictures are patients' numbers and have not any meaning. Positive controls were *E. faecalis* 29212 (Standard for *E.faecalis), E. faecium* C38 (Standard for *E. faecium*) and *E. faecalis* MMH594 (Standard for *esp*).

faecalis previously [17], but not in other *Enterococci* [18]. We revealed high frequency of *esp* genes in both *E. faecium* and *E. faecalis* (76.1%, 77.9% respectively) isolated from urinary tract infection. It was higher rate than reported in previous studies on clinical samples [17, 19]. *esp* has been reported as the first important gene for biofilm formation in *Enterococci* [6, 9]. The ability to form biofilm on abiotic surfaces is considered to be an important virulence property of the *Enterococci* [20], biofilm is an assemblage of microbial cells associated with a surface and enclosed in a matrix of primarily polysaccharide material [l6]. Defined architecture of the biofilm provides an optimal environment for the exchange of genetic material between bacteria and increases the innate resistance of the bacterium to antibiotic and activities of the host immune response [6, 21]. *esp* gene positive isolates were considered as expressing the *esp* protein [9, 22]. Results of this study shows importance of the biofilm formation and the presence of *esp* in clinical samples, especially in the urinary tract infections. The nature of urinary tract infections necessitate biofilm for bacteria, and presence of *esp* in most of urinary cultured *Enterococci* endorse importance of this gene for survival of bacteria in urinary tract. It has been also demonstrated that *esp* increased the hydrophobicity of bacterial surface [23]. Therefore, it can help *Enterococci* to match with urinary tract condition.

In this study, we found significant correlation between the presence of *esp* and the antibiotic resistance. Our data

showed significant correlation between presence of *esp* gene and resistance to Vancomycin in *E. faecium* (Fig. **1**) and resistance to Ampicillin, Chloramphenicol and Tetracycline ($p<0.001$, $p<0.001$, $p<0.001$) (Fig. **2**) in *E. faecalis.* In a study by Billstrom *et al*, they revealed strong correlation between *esp* carriage and antimicrobial resistance to Ampicillin and Ciprofloxacin and Imipenem in clinical blood culture isolates of *E. faecium* [24]. But in our urinary tract isolates of *E. faecium*, we didn't find similar correlation (Fig. **1**). However, we found significant correlation with resistance to Vancomycin and presence of *esp*. Also in *E. faecalis* isolates we had significant correlation between the presence of *esp* and resistance to the Ampicillin, Tetracycline and Chloramphenicol (Fig. **2**).

In conclusion, our finding provides additional evidence for the presence of *esp* in *Enterococcus* isolates from urinary tract infection and high rate of antibiotic resistance may lead to strong biofilm formers and strong adherent to host cells. These three factors may play an important role in enterococcal infections. The presence of these bacteria in urinary tract infection in addition to its multi resistance, close attention should be given to these strains in order to reduce risk for development of disease caused by *Enterococci* in other areas of the body. Furthermore, additional studies about role of *esp* expression on biofilm formation of enterococci is needed.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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