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Evaluation of the Frequency of Virulence Genes in Enterococcus Faecalis Isolates from Fecal Samples by Multiplex PCR Method

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ABSTRACT

BACKGROUND AND OBJECTIVE: Enterococcus faecalis, as the leading cause of hospital-acquired infections, contains virulence factors which are involved in bacterial colonization, immune system resistance, competition with other microorganisms, and damage to the host through the production of secretory factors. The aim of this study was to evaluate the frequency of virulence genes in Enterococcus faecalis isolates obtained from fecal samples by multiplex polymerase chain reaction (PCR) method.

METHODS: In this descriptive-analytical study, 200 fecal samples were collected from Kerman medical centers, and 60 Enterococcus faecalis isolates were identified by culturing in selective media (e.g., KF Streptococcus agar) and performing biochemical tests. Multiplex PCR method was used to identify virulence genes.

FINDINGS: Based on the findings, the frequency of Enterococcus faecalis infection was 52% and 48% among females and males, respectively; overall, the frequency of virulence genes was higher in females than males. Also, according to the results, asa1 (80.6%) and cylA (16.1%) genes had the highest and lowest frequencies, respectively, whereas hyl gene was not detectable in any of the samples.

CONCLUSION: As the findings revealed, the frequency of Enterococcus faecalis infection was higher in the female population. Considering the anatomy of female urinary tract and the subsequent recurrent infections in women, the frequency of virulence genes was higher in women.

KEY WORDS: *Enterococcus faecalis, Virulence genes, Polymerase chain reaction*.

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Introduction

Enterococci are Gram-positive facultative anaerobic bacteria, which are part of the normal intestinal flora and are regarded as important pathogens, particularly in hospital-acquired infections (1). The success of these bacteria in the induction of hospital-acquired infections depends on virulence factors, which are involved in the colonization of microorganisms, resistance to the immune system, competition with other microorganisms, and damage to the host through the production of secretory factors. Enterococci can cause serious infections such as bacteremia, septicemia, urinary tract infection, meningitis, and wound infection, especially in cases with skin damage, immunosuppression, prolonged hospital stay, and even indiscriminate use of antibiotics (2, 3). Overall, Enterococci show less virulence potential, compared to other bacteria such as Streptococcus and Staphylococcus (4).

Previous research has revealed the presence of virulence factors in Enterococci, each with a specific role in disease pathogenesis (5). Virulence factors such as hemolysin/cytolysin, which can be found in nearly 30% of Enterococcus faecalis samples, are encoded on pheromone-responsive plasmids or pathogenicity islands and induce lysis in red and white blood cells (6). Besides damaging the host tissues and reducing the immune system response, gelatinase is involved in the activation of autolysins, destruction of peptidoglycans (followed by DNA release), and biofilm formation (7). In addition, hyaluronidase leads to the destruction of hyaluronic acids and plays an important role in the proliferation of bacteria at the site of primary infection (8). Aggregation substance, which carries asa1 gene in Enterococci and is produced in response to sex pheromones, can trigger adhesion among bacteria and induce the formation of cell masses. Furthermore, this substance is known to play an important role in the induction of cell adhesion, cell invasion, and destruction of myocardial and lung tissues (9-11). Enterococcal surface protein (ESP) has similar functions to the aggregation substance (12). The factor responsible for collagen binding is also involved in the binding of bacterium to collagen and laminin, and its mutation reduces endocarditis and urinary tract infections (13).

According to a previous study by Richards et al., Enterococci play a major role in causing urinary tract, surgical, and blood infections (14). Vancomycin-resistant E. faecalis is the predominant species involved in these infections (15). In this regard, in a previous study, the highest frequency of gelatinase and hemolysin was reported among Enterococci (16). E. faecalis, containing gelatinase, has been identified in nearly 93% of patients with endocarditis (17). According to the literature, cytolysin and gelatinase were found in 29% and 48% of children with E. faecalis in Norway, respectively (18).

Moreover, Eaton and colleagues showed that virulence genes are more frequently found in E. faecalis isolates from clinical samples, compared to isolates from food samples (19). On the other hand, in a previous study in Iran, no single factor was identified as the dominant predictor of virulence, and the induced damage was mostly attributed to the cumulative effects of various factors (20). Based on various studies in different countries, esp and hyl genes are identified as the most frequent virulence genes (21). In a study by Chow et al., cytolysin was mainly isolated from patients with endocarditis (22). Also, hemolysin has been proposed as a virulence factor in animal models. In this regard, in a previous study on rats, the highest mortality rate was reported in strains containing hemolysin (cytolysin) (23).

Kreft et al. revealed the involvement of the cumulative factor in the binding of Enterococci to hepatic cells and hypothesized that this factor might be involved in the pathogenesis of these bacteria (24). Also, in a research study conducted in Iran, the cumulative factor was more commonly reported in the isolated clinical samples, compared to the control isolates (25). Based on the aforementioned background, the aim of this study was to evaluate some virulence factors and determine the frequency of some genes (cylA, gelE, asa1, hyl, and esp) in E. faecalis isolates, obtained from fecal samples through multiplex polymerase chain reaction (PCR) method.

Methods

Sample collection and isolation: In this descriptive-analytical study, by using the sample size formula (95% confidence level, acceptable probability of error: 0.05), 200 fecal samples were collected from patients, referring to healthcare centers of Kerman, Iran in June-December, 2014. The samples were transferred to blood agar, Macconkey agar, and KF Streptococcus Agar media for culturing and were incubated at 37°C for 24 h. The cultured colonies were identified using biochemical tests, including esculin hydrolysis test,

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growth on sodium chloride (NaCl) medium, hippurate hydrolysis test, and glucose fermentation. Based on the evaluations, 60 E. faecalis isolates were confirmed. Morphological and biochemical characteristics of the bacteria were evaluated to confirm E. faecalis isolates. The obtained samples were investigated in selective media and confirmed by biochemical tests. Samples with biochemical characteristics indicative of E. faecalis were recognized as positive (26).

DNA extraction and multiplex PCR test: For DNA extraction, CinnaGen Gram-positive bacteria extraction kit (CinnaPure DNA Kit, PR881614) was used. Following DNA extraction, its concentration was measured, using a photometer device (Eppendorf), and then, multiplex PCR test was performed (table 1). Initial denaturation was performed at 95°C for 5 min, followed by denaturation at 95°C for 1 min, annealing at 56°C for 1 min, 35 cycles of extension at 72°C for 1 min, and final extension at 72°C for 5 min (27). For multiplex PCR reaction, 9.6 ml of distilled water, 2 ml of 10X PCR buffer, 0.8 ml of 1.5mM $MgCl_2$, 0.8 ml of 5 Mm deoxynucleotide (dNTP) solution mix, 1.5 ml of each primer (concentration: 10 pm/µl), 0.3 ml of Taq DNA polymerase (2.5 U), and 4 ml of DNA sample were used in a final volume of 25 ml (27). Multiplex PCR test was performed in Techne device. For the evaluation of Multiplex PCR products, the samples were transferred to 1% agarose gel and studied in Gel Doc device (Bio-Rad Laboratories) after staining.

Results

Multiplex PCR results showed that among 60 studied isolates, the most frequent genes were asa1 (n=45, 75%), gelE (n=34, 56.6%), esp (n=17, 28.3%), and cylA (n=7, 11.6%), respectively; however, hyl gene was not detected in any of the isolates. Furthermore, in terms of gender, the frequency of E. faecalis virulence genes was the highest in the female population (52% in females vs. 48% in males); as the findings revealed, the difference was statistically significant (fig 1). Also, molecular identification of genes and PCR products with respect to product length(bp) is presented in figure 2.

Table 1. The sequence of primers used in this study

Gene	Virulence factor	Primer	Nucleotide sequence (3' to 5')	Product length(bp)	Source
asa1	Aggregation substance	ASA1-F	GCACGCTATTACGAACTATGA	375	(27)
		ASA1-R	TAAGAAAGAACATCACCACGA		
gelE	Gelatinase	GEL-F	TATGACAATGCTTTTTGGGAT	213	(27)
		GEL-R	AGATGCACCCGAAATAATATA		
cylA	Cytolysin	CYT-F	ACTCGGGGATTGATAGGC	688	(17)
		CYT-R	GCTGCTAAAGCTGCGCTT		
esp	Enterococcal surface protein (ESP)	ESP-F	AGATTTCATCTTTGATTCTTGG	510	(28)
		ESP-R	AATTGATTCTTTAGCATCTGG		
hyl	Hyaluronidase	HYL-F	ACAGAAGAGCTGCAGGAAATG	276	(8)
		HYL-R	GACTGACGTCCAAGTTTCCAA		

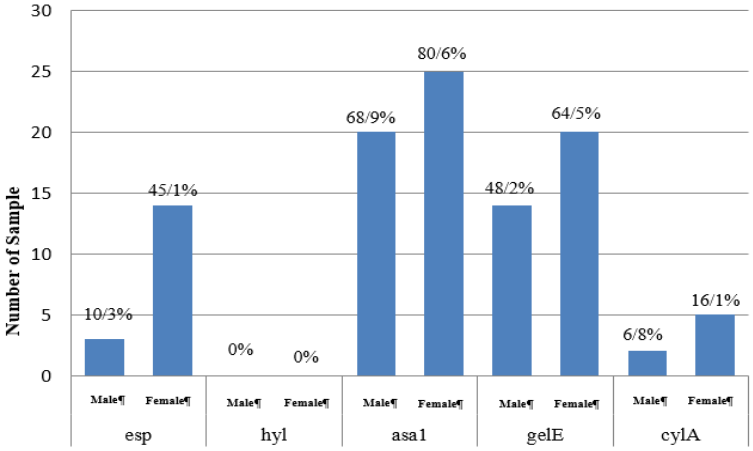

Figure 1. Frequency distribution of Enterococcus faecalis virulence genes in terms of gender

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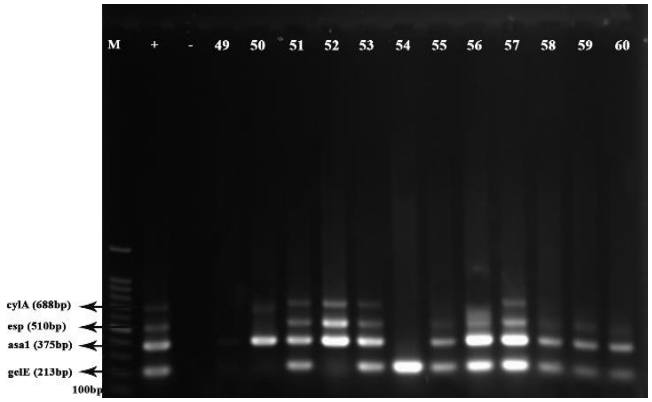

Figure 2. PCR results on a number of isolates (from left to right: 100 bp marker, positive control, negative control, isolated samples, and band length of each gene, respectively)

Discussion

Based on the present findings, the frequency of E. faecalis infection was 52% among females, which was higher than the rate reported in males. It should be noted that the infection rate plays a major role in disease pathogenicity, particularly in women. Also, in this study, the frequency of virulence genes was higher among females, compared to males. It seems that one of the contributing factors for this difference is the frequent consumption of antibiotics in women, which leads to the higher frequency of E. faecalis and consequently the virulence genes. Virulence factors in E. faecalis include the aggregation substance, ESP, hemolysin (hyl) with bactericidal and hemolytic activities, and gelatinase enzyme (11).

Based on the present findings and the frequency of virulence genes in E. faecalis, the active role of these genes in the pathogenesis of E. faecalis infection was confirmed. Given the fact that no protein toxins have been identified in Enterococci, bacterial virulence is probably caused by a cluster of factors and enzymes (produced by bacteria), antibiotic resistance, and the cumulative factor involved in plasmid exchange (29). The results of a study by Mozafari et al. showed that the frequency of virulence factors, such as hemolysin, gelatinase, hemagglutinin, desoxyribonuclease, pheromone production, and cumulative factor, was higher in E. faecalis samples, compared to E. faecium (25). Moreover, in a study by Ghasemi et al., 19 gelatinase strains were detected among 95 E. faecalis samples. Based on the reported findings, 42 and 53 strains were hemolytic and non-hemolytic, respectively. Overall, no single dominant factor was identified as an important predictor of virulence, and a combination of factors seems to be involved (20). According to the literature, the cumulative effects of genes are more significant than their independent role. In a previous study conducted by Padmasini et al., in a total of 157 Enterococcus isolates obtained from different hospital wards, 73 (46.5%) were E. faecalis. Based on the results, cyla, gelE, hyl, asa1, and esp genes were detected in 23.6%, 51.6%, 6.4%, 55.4%, and 49.7% of the isolates, respectively (30); the reported findings were in line with the present results in terms of the frequency of some genes.

Moreover, the low frequency of hyl gene, which was in consistence with previous studies, can be justified. In a study by Vankerckhoven et al., 135 out of 271 E. faecalis isolates showed drug resistance. The frequency of esp and hyl genes was 73% and 29% in fecal samples, respectively (27); interestingly, hyl gene was present in none of the studied samples. Also, the results related to the frequency of esp gene in samples isolated from women and men were in congruence with the study by Padmasini and colleagues. A study by Worth et al. indicated that esp gene was present in 81.5% of the studied patients. However, no relationship was observed between the frequency of complications and the presence of esp gene in these patients (31). In 2010, Biendo et al. concluded that 29.8% of the samples were positive for hyl gene, while 70.2% were positive for both esp and hyl genes, based on PCR test results (21); however, in the current study, these two genes were not simultaneously detected. ESP, which is encoded by the chromosomal esp gene, is associated with increased virulence, colonization, urinary tract stability, and biofilm formation. Moreover, this protein is involved in the initial adhesion and biofilm formation in E. faecalis. The role of this protein in the colonization and survival of E. faecalis in urinary tract infections has been demonstrated in animal models (9, 12, 32).

Large amounts of ESP can be mostly found in endocarditis and bacteremia isolates, while rarely seen in healthy human stool samples. Among the studied genes, the frequency of esp gene was estimated at 28% among women, which was compatible with earlier findings and the role of this gene in the development of urinary tract infections.

Also, in the present study, the overall frequency of this gene was estimated at 28.3% in both genders, and the gene was detected in 17 out of 60 isolates. Also, in a study by Hassani et al., among 220 samples, 65.9%, 49.5%, and 53.6% were positive for gelE, esp, and

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asa1 genes, respectively (4). Gelatinase enzyme is encoded by the chromosomal gelE gene. This enzyme serves as an extracellular metalloprotease, which hydrolyzes collagens, gelatins, and small peptides, and is involved in the development of endocarditis in animal models. Biofilm formation allows cells to survive under unfavorable conditions (19, 23). In the presents study, the frequency of gelE gene was the highest (56.6%) among the studied genes, given its role in the stability of bacteria caused by biofilm formation; also, its frequency was estimated at 37.7% among female patients. Overall, cytolysin production significantly intensifies the severity of endocarditis and endophthalmitis in animal models and increases the intensity of Enterococcal infections in humans (6, 33). Epidemiological studies have confirmed the role of cytolysin in the incidence of such conditions. In a previous study by Coque et al., there was no significant difference regarding cytolysin gene expression among E. faecalis isolates separated from endocarditis, bacteremia, and stool samples of healthy individuals (17). Moreover, in another study, only 16% of E. faecalis isolates were able to produce cytolysin, and the role of this protein was confirmed as an important virulence factor (29). Therefore, the low frequency of cytolysin gene in the present study can be justified (11.6% in total and 9.4% in females). In consistence with our results, cytolysin gene in clinical E. faecalis isolates might indicate a negative phenotype pattern which lacks hemolytic activity (as genetically confirmed). However, it should be noted that environmental factors such as changes in the infection site might trigger the activity of genes (34).

In the present study, the frequency of E. faecalis infection was higher in the female population, which is due to the physiological anatomy of female urinary tract and higher incidence of urinary tract infections among women (therefore, high frequency of virulence genes). Also, the incidence of infection, induced by a combination of virulence genes, was higher than single genes. The pathogenesis of E. faecalis can be explained by two important factors. First, E. faecalis can survive in different environments and infect many hosts. Second, this bacterium contains various virulence factors, which are genetically transmitted from one generation to another. Consequently, by the use of methods for the detection of virulent strains, we can design preventive strategies and control the spread of these strains.

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