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Evaluation of Virulence Factors and Plasmid-Related Transmissibility among Different Isolates of *Enterococci*

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ABSTRACT

The incidence of virulence factors among 114 *Enterococcus faecalis* and 35 *Enterococcus faecium* strains from different clinical specimens were compared with those isolated from control groups. A few of the isolates expressed two or more of the following traits simultaneously: hemolysin, aggregation substance, gelatinase, DNase, hemagglutinin and antibiotic resistance. The frequencies of hemolysin, aggregation substance, and gelatinase production in *E. faecalis* were much higher than those in *E. faecium*. However, no statistically significant differences were detected in the other traits. Two of the isolates showed total resistance to all of the antibiotics tested, and others displayed varied degrees of resistance pattern. The frequency of plasmid transfer was shown to be 10^{-4}- 10^{-7} per donor among the isolated strains. The plasmid profile of the bacteria indicated that most of the isolates contained one or more plasmids with molecular weight ranging in 2 to 42 Mda regions. Resistance to gentamicin and tetracycline was the most observed antibiotic resistance pattern, and had the property of efficient inter-enterococcal spp. transfer by mating. *Iran. Biomed. J. 9 (2): 51-55, 2005*

Keywords: Pheromone, Mating, Virulence factors, Enterococci

INTRODUCTION

The vast preponderance of enterococcal existence is as a commensal organism in gastrointestinal tract of humans. Over the past two decades, enterococcal strains have emerged that are resistant to virtually all antibiotics, including what for many infections such as subacute bacterial endocarditis, was the antibiotic of last resort, vancomycin [1]. Under most circumstances, enterococci stably co-exist with the host. However in the era of modern medicine, situation now routinely arise when this balanced commensalisms is disrupted. Because of the increasing difficulty in treating enterococcal infection, more effort is being devoted to understanding factors that undermine the commensal relationship, with a view toward targeting these factors with new therapeutics [2].

In addition to antibiotic resistance, other virulence factors include the phenotypic markers, gelatinase, hemolysin DNase, and aggregation substance protein [3]. Even though Well and Maddaus [4] have demonstrated enterococcal invasiveness in the murine orogastric-feeding model; its pathogenic contribution in human is rather vague. Pheromone responsive transmissible plasmids are common among strains of *E. faecalis*. Conjugative transfer of these plasmids is induced by peptide sex pheromones elaborated by the potential recipient cells [5]. Donor cells in response to this pheromone express a surface protein, called aggregation substance, that enables the donor and recipient cells to adhere and transfer plasmids by conjugation [6]. Hemolysin is a cytolytic protein capable of lysing human, horse, rabbit, but not sheep erythrocytes. It also possesses bacteriocins activity against many Gram-positive bacteria. Hemolysin, aggregation substance and antibiotic resistance in enterococci are transferred by transposons or plasmids that code them. The gene that codes for hemolysin is usually plasmid borne, although it can be located on the chromosome as well [7]. Using animal models, Ike *et al.* [8] showed that *E. faecalis* strains expressing cytolysine activity are 10 times more toxic than non-cytolytic strains.

Enterococcal gelatinase can hydrolyze gelatin, collagen, casein, hemoglobin and other bioactive peptides. It also causes direct and indirect damage

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to host tissue and facilitate microbial invasion and survival in the host [9]. A later study from Makinen [10], renamed this *E. faecalis* metallopeptidase as "Coccolysin" based on its ability to inactivate human endothelin (a vasoactive peptide). In addition to aggregation substance, hemagglutinin may also contribute to the attachment of the bacterial cells to host and therefore presumably be involved in pathogenesis [11]. Deoxyribonuclease is another factor that may be involved in bacterial virulence. Despite many investigations conducted on the enterococci DNase, its exact role in the disease process is rather controversial. A number of virulence factors that have been described to date and enhance the virulence of enterococci in animal models are investigated in this study to evaluate a possible linkage between the presence of these factors and human infections.

MATERIALS AND METHODS

Bacterial strains, media and reagents. Enterococcal strains (116:1 isolated per patient) were isolated from different clinical samples such as urine and wounds. A majority of the samples were urine specimens (109 isolates) and the others were isolated from wounds (7 samples). The samples were collected during February 2002 and May 2003. A control group (37:1 isolate per person) collected from feces of healthy individuals was similarly investigated. The clinical samples related to infective endocarditis, bacteremia and urinary tract infection were collected from patients admitted to Kasra Hospital (Tehran, Iran). The isolates were identified based on phenotypic characterization described before [12], and out of 153 isolates 112 (75%) were identified as *E. faecalis*, 40 (26%) as *E. faecium*, and 2 (1.3%) as *E. mundti*. The sex pheromone-producing plasmid free *E. faecalis* JH2-2 was selected as the recipient. The type of the strains used to devise the identification scheme was *E. faecalis* ATCC 19433. Hemolysin production was determined by plating enterococci onto Todd-Hewitt agar (Difco), supplemented with 5% human blood and incubated at 37ºC in a CO_2 chamber for 48 h. A clear zone of β-hemolysis was considered as a positive result. Gelatinase production was tested using Todd-Hewitt agar containing gelatin (30 g lit^{-1}; Difco) at 37°C for 48 h. Appearance of clear zone around the colonies was considered to be a positive indication of gelatinase production. Hemagglutination test was performed with human group A (3%) erythrocytes according to the protocol described elsewhere [11]. For detection of DNase, bacterial isolates were cultured on DNase agar (Oxoid, UK) and after incubation at 37°C for 48 h and after flooding the plates with 1N HCl, halo zones were seen around colonies [13]. Production of aggregation substance was examined by induction of clumping. In summary, 200 µl of an 18 h culture supernatant of the pheromone-producing *E. faecalis* JH2-2 strain, grown in Todd-Hewitt broth, was added onto the enterococcal strain being tested (0.5%). After incubation at 37ºC for 2, 4, 8 and 24 h, cell clumping was directly visualized by naked eye.

Susceptibility testing. All enterococcal strains were tested by disk diffusion and dilution tests according to the NCCLS recommendations [14]. The antibiotic concentration per disk was as follows: vancomycin (30 µg), erythromycin (15 µg) tetracycline (30 µg), penicillin-G (10 units) and chloramphenicol (30 µg). Minimal inhibitory concentration (MIC) was determined against a range of antimicrobial agents including 0.5 to 128 µg vancomycin ml^{-1} (Sigma, Germany), 0.25 to 32 µg erythromycin ml^{-1} (Hi-media, India), 250 to 1,000 µg gentamicin ml^{-1} (Sigma, Germany), 0.5 to 32 µg tetracycline ml^{-1} (Sigma, Germany), 0.5 to 8 µg rifampicin ml^{-1} (Hi-media, India) and 4 to 64 µg penicillin G ml^{-1} (Jaber-ebn-e-Hayyan, Iran).

Plasmid analysis and conjugation experiments. Enterococcal plasmids were isolated by a modified alkaline lysis procedures [15]. Cell suspension was incubated with lysozyme (50 mg ml^{-1}) in 10 mM Tris-HCl, 1 mM EDTA (pH. 8.0), containing 50 mM glucose at 37°C for 1 h. Approximate molecular weights of the isolated plasmids were determined by comparison with known plasmids isolated from control strains *Escherichia coli* 39R861 and V517. Plasmid DNA isolated from all strains was separated by electrophoresis on 0.7% agarose gel using 0.5 X TBE buffer and run at 90 V, for 1-2 h. Fractionated plasmid DNA was visualized under UV light in gel document (Vilber Lourmat Company, Japan) following staining with ethidium bromide. Conjugation experiments were carried out using broth mating and filter mating techniques between plasmid free-pheromone producing strain *E. faecalis* JH2-2 (fusidic acid and rifampicin resistant) and clinical isolates that were susceptible to rifampicin [16]. A few investigators have indicated that erythromycin resistance can be transferred without the involvement of any plasmid DNA in transconjugants [16]. For this reason, an enterococcal strain that contained resistance to all antibiotics and was devoid of any plasmid DNA,

Table 1. Incidence of virulence factors among enterococcal isolates.

Virulence factor	Group I*			Group II**		
	E. faecalis	*E. faecium*	Incidence	*E. faecalis*	*E. faecium*	Incidence
Clumping phenotype	20 (23.3%)	1 (9.0%)	18.1 %	2 (9.0%)	0 (0.0%)	5.4%
Hemolysin	16 (17.2%)	3 (12.0%)	16.4 %	3 (12.6%)	2 (12.4%)	13.5%
Gelatinase	12 (13.3%)	2 (8.0%)	12.0 %	6 (26.0%)	0 (0.0%)	16.2%
DNase	1 (1.1%)	2 (8.0%)	2.6 %	1 (4.3%)	1 (7.1%)	5.4%
Hemagglutination	88 (97.7%)	21 (91.3%)	94.0 %	23 (92.0%)	10 (75.0%)	89.2%
Hly and Agg[§]	2 (2.2%)	1 (4.3%)	2.6 %	1 (4.0%)	0 (0.0%)	2.7%
Hly , Agg and DNase	0 (0.0%)	1 (4.3%)	0.8 %	0 (0.0%)	0 (0.0%)	0.0%
Agg and Gel[ξ]	1 (1.1%)	0 (0.0%)	0.8 %	1 (7.1%)	0 (0.0%)	2.7%

*, clinical isolates; **, healthy volunteer's isolates; [§], hemolysin, aggregation substance; [ξ], gelatinase

transconjugants were retested for growth on plates containing tetracycline (16μg ml^{-1}), gentamicin (500μg ml^{-1}), vancomycin (50 μg ml^{-1}), rifampicin (50 μg ml^{-1}) and penicillin G (16 μg ml^{-1}) according to the corresponding MIC for these strains that had been determined in this study. Plasmid profile of the transconjugants and the conjugation frequencies were determined.

Restriction enzyme analysis of plasmid DNA. For comparison of the residing plasmids in the donor as well as the transconjugate cells, the plasmid DNA was digested with *Hin*dIII restriction endonuclease according to the manufacturer's instruction (Fermentas, Lithuania). The digested DNA was subsequently electrophoresed on agarose gel.

RESULTS AND DISCUSSION

Out of the 153 enterococcal isolates from 116 patients (group I) and 37 healthy individuals (group II); 90 (77.0%) and 23 (6.2%) were *E. faecalis*, 25 (21.5%) and 14 (38.0%) were *E. faecium*, respectively. In group I, two isolates (1.5%) were identified as *E. mundtii* and produced yellow pigment. Between *E. faecalis* and *E. faecium*, some strains produced β- hemolysin, gelatinase, DNase and aggregation substance.

Most of the isolates produced hemagglutinin (Table 1). Coque *et al.* [7] also showed that as many as 13% of the infection-derived *E. faecalis* urine isolates were hemolytic, compared to 20% of the stool specimens from healthy volunteers. Gelatinase production has been implicated in the virulence of *E. faecalis* in an animal model [17]. Coque *et al.* reported a significantly higher frequency of gelatinase positive phenotype in clinical isolates (54% of endocarditis isolates, 58% of non-endocarditis clinical isolates) compared with fecal isolates from healthy volunteers (28%). Pathogenesis studies show that aggregation substance and cytolysin act synergistically to enhance virulence by facilitating achievement of quorum-sensing model of cytolysin regulation, resulting in tissue damage and potentially deeper tissue invasion [1].

In our study, aggregation substance observed in some of hemolysin positive isolates and co-expression of aggregation substance and gelatinase phenotypes was seen in two cases. In contrast to the results of most investigations, we found 5 (3.3%) DNase positive strains in both *E. faecalis* and *E. faecium*. Hemagglutinin, which contributes to bacterial attachment to host cells, was the most prevalent virulence factors detected in our strains (92%).

As shown in Table 2, antibiotic resistance in the control group (group II) was much lower than the other groups. This study indicated that both *E. faecalis* and *E. faecium* isolates carried antibiotic resistance markers. Ten isolates including *E. faecium* [4] and *E. faecalis* [6] have displayed multi-drug resistance. Two isolates were also resistance to all of the antibiotics tested. The MIC for penicillin G in all the resistant isolate was 32 μg ml^{-1}; except for two of the bacteria which was greater than 64 μg ml^{-1}. The MIC for tetracycline in all the resistant strains was 16 μg ml^{-1}; except in two of the isolates which was greater than 64 μg ml^{-1}. The MIC for gentamicin and erythromycin was greater than 1,000 μg ml^{-1} and 32 μg ml^{-1}, respectively.

The two vancomycin resistant enterococcal strains displayed MIC values at 64 μg ml^{-1} and 128μg ml^{-1}. Each of the eight enterococcal strains, *E. faecalis* [6] and *E. faecium* [2] that were chosen for the conjugation experiment possessed a 42 Mda plasmid. Two strains were also in possession of a 98 Mda plasmid and two carried a 20 Mda plasmid. Additionally, plasmids of approximately 22, 7, 3 Mda and 2 Mda were also detected in these isolates (Fig. 1).

Almost similar results were obtained in both mating protocols, except for gentamicin and tetracycline which more transconjugate cells

Table 2. Resistance of *E. faecalis* and *E. faecium* strains to antibiotics.

Antibiotic[++]	Group I[*]		Group II[**]	
	E. faecalis	***E. faecium***	***E. faecalis***	***E. faecium***
Vancomycin	1 (1.1%)	1 (4.4%)	0 (0.0%)	0 (0.0%)
Gentamicin	19 (21.1%)	7 (28.0%)	2 (9.5%)	0 (0.0%)
Tetracycline	53 (59.0%)	5 (20.0%)	3 (14.3%)	1 (4.7%)
Penicillin G	23 (26.0%)	7 (30.0%)	6 (28.5%)	2 (9.5%)
Erythromycin	47 (52.2%)	14 (56.0%)	4 (19.0%)	0 (0.0%)

*, clinical isolates; **, healthy volunteer's isolates; [++], Resistance interpreted on the basis of MIC for Enterococci conducted according to the NCCLS guidelines; MIC: High-level gentamicin greater than 500 µg ml^{-1}; tetracycline greater than 16 µg ml^{-1}; penicillin G greater than 16 µg ml^{-1}; erythromycin greater than 8 µg ml^{-1}; and vancomycin greater than 32 µg ml^{-1}.

were detected by the filter mating method. Antibiotic resistance was co-transferable in most cells. In one strain, transconjugants acquired all of the resistant traits. The gentamicin and tetracycline resistant were the most efficient conjugationally transferable genes. The frequency of transfer was 4 $\times 10^{-4}$ to 4 $\times 10^{-7}$ per donor. The 42-Mda plasmid was acquired by one transconjugate, and in two transconjugants, the 20 Mda plasmid was received. In addition to these plasmids, most transconjugants also acquired the 2, 3 and 6 Mda plasmids; either individually or all together.

One resistant strain that lacked any plasmid was also capable of generating transconjugants. One of the trans-conjugants acquired simultaneous resistant to vancomycin and gentamicin. Vancomycin and tetracycline resistance was also co-transferred in one cell. Complete digestion of the donor as well as the conjugated plasmids with *Hin*dIII produced similar DNA fragments. Hemolysin and aggregation phenotypes were detected more frequently in *E. faecalis.* This may be due to the fact that, genes for these two factors are being carried on pheromone-responsive plasmids such as pAD1 that is possessed solely by *E. faecalis* [18].

The DNase producing enterococcal strains were very few and it does not seem to be a statistically significant difference between the clinical and control isolates. The frequency of gelatinase producing strains was also very low but was more often detected in *E. faecalis*.

It is clear that in the hospital environment, antibiotic usage may influence selection of pathogenic enterococci, which may lead to infections or super infections. Multiple drug resistance is a growing problematic phenomenon, especially to vancomycin and to the synergistic action of β-lactams and amino-glycosides, which leave few therapeutic options [19]. In this study, we found significant differences between the control and clinical isolates, but did not observe this among *E. faecium* and *E. faecalis* strains. Since high-level gentamicin resistance correlates with loss of synergistic bactericidal activity between cell wall acting drug and all currently available aminoglycosides; the prevalence of highly gentamicin-resistant strains in diverse geographic areas is of clinical concern. In this study, all of resistant isolates showed MIC greater than 1,000 µg ml^{-1} for gentamicin, however three of them failed to transfer this trait upon mating. Resistance markers were also able to transfer simultaneously and in two strains, resistance to tetracycline and gentamicin was co-transfer with hemolysin. Additionally, we

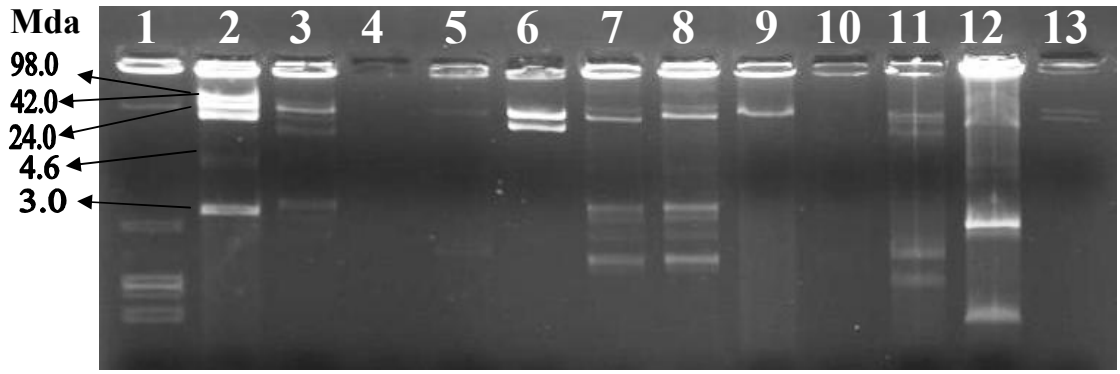

Fig. 1. Agarose gel electrophoresis of plasmid DNase from enterococcal strains. Lane 1 and Lane 2, plasmid profile from *E. coli* V517 and *E. coli* 39R861, respectively that were used as size marker; Lane 3 to 13 representative enterococcal isolates with plasmids that code resistance and some of virulence factors such as cytolysine.

obtained a DNase positive transconjugant that was not reported by any investigator before.

Although DNase phenotype was transferable among the clinical enterococcal isolates in this study, the clinical significance of this finding requires further investigation. It must be considered that in some of our clinical specimens, other potentially pathogenic bacteria such as coagulase negative *Staphylococci* and *E. coli* were also present. Recent studies indicated that gene transfer from *E. faecalis* to *Staphylococci* can occur readily and the transferred plasmids were stable in the transconjugants [1]. Thus, further genetic experiments are needed to determine the possibility of inter-species gene transfer in clinical samples and their possible contribution in the disease process.

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