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Survey of Virulence Determinants among Vancomycin Resistant *Enterococcus faecalis* and *Enterococcus faecium* Isolated from Clinical Specimens of Hospitalized Patients of North west of Iran

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Abstract: Recent data indicates an increasing rate of vancomycin resistance in clinical enterococcal isolates worldwide. The nosocomial enterococci are likely to harbor virulence elements that increase their ability to colonize hospitalized patients. The aim of this study was to characterize virulence determinants in vancomycin-resistant enterococci (VRE) obtained from various clinical sources.

During the years 2008 to 2010, a total of 48 VRE isolates were obtained from three University teaching hospitals in Northwest, Iran. Initially, phenotypic speciation was done and minimum inhibitory concentrations (MICs) of vancomycin were determined by agar dilution method and E-test. Then, species identification and resistance genotypes along with detection of virulence genes (*asa1*, *esp*, *gelE*, *ace* and *cpd*) of the isolates were performed by multiplex PCR.

Thirty eight isolates were identified as vancomycin-resistant *Enterococcus faecium* (VREfm) and ten as *E. faecalis* (VREfs). Irrespective of the species, *vanA* gene (89.58%) was dominant and three phenotypically vancomycin susceptible *E. faecium* isolates carried the *vanB* gene. Among virulence genes investigated, the *esp* was found in 27(71%) VREfm strains, but did not in any VREfs. Other virulence determinants were highly detected in VREfs strains. Our data indicate a high prevalence of *E. faecium* harboring vancomycin resistance with *vanA* genotype and the two VRE species displayed different virulence genes.

Keywords: *Enterococcus faecalis, Enterococcus faecium*, Vancomycin resistance, Virulence gene.

INTRODUCTION

Enterococci are Gram-positive intestinal commensals of humans and other animals, in addition to be an isolate from environmental sources. During the past decades an enhancement in the prevalence of enterococal infections such as bacteremia and urinary tract infections along with emergence of multi antimicrobial resistance, particularly VRE has been reported worldwide [1, 2].

Among vancomycin-resistance phenotypes in enterococci, VanA and VanB possess highest clinical importance. Strains resistant to vancomycin and teicoplanin were assigned to VanA phenotype while, those susceptible to teicoplanin but resistant to vancomycin were considered as the VanB phenotype [2]. The *vanA* and *vanB* gene clusters contain nine different genes for a two-component regulatory system (*vanR* and *vanS*), three resistance genes (*vanH*, encoding dehydrogenase; *vanA* or *vanB*, encoding ligase; *vanX*, encoding DD-dipeptidase); an accessory gene (*vanY*); and the *vanZ* gene, which is present in the *vanA* gene cluster whereas, the *vanW* gene is found only in the *vanB* operon. VanA is carried on Tn*1546* whilst, VanB on transposons Tn*1547* and Tn*1549*, which may be found on plasmids or inserted on the chromosome [3].

Despite the fact that *E. faecalis* has been observed as the predominant species in clinical infections, an increase in the prevalence of *E. faecium* has been seen recently [4]. This shift is likely to be explained in part by the emergence of vancomycin resistant enterococci (VRE) and *E. faecium* being the dominant detectable species among them [5].

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Unfortunately, little is known about the pathogenic mechanisms or virulence factors of this microorganism [6]. Enterococcal infections may occur *via* the ability to persist on harsh environments due to their intrinsic properties and even these conditions may contribute largely to emergence of this organism as a nosocomial pathogen [7, 8]. The process of invasion is usually facilitated by damage to host tissues and the presence of bacterial virulence factors such as adhesins along with antibiotic resistance that gradually assist in advancement and furtive survival in newly infected place. At the next, enterococci utilize several virulence factors for adherence and colonization in the infection site by formation of cell aggregates such as biofilms [5, 9]. At the end, interactions between host and enterococci leads to the clinical manifestation of infection in target vital tissues [5].

By consideration of the fact that the presence of putative virulence determinants among clinical isolates may promote emergence of infections and persistence of enterococci in nosocomial locations causes an increase in antibiotic resistance and act as facilities for emerging infections [10, 11], this study was designed to survey distribution of virulence genes including *asa1*(aggregation substance), *esp* (Enterococcal surface protein), *gelE* (gelatinase), *ace* (collagen adhesion) and *cpd* (sex pheromones determinant) in clinical isolates of vancomycin resistant *E. faecalis* and *E. faecium* at molecular level. To our knowledge, this is the first report from the Northwest of Iran.

MATERIALS AND METHODOLOGY

VRE Isolates and Susceptibility Tests

Thirty-eight VREfm and 10 VREfs isolates, obtained from different clinical specimens submitted in three University teaching hospitals of Tabriz (Imam Reza and Sina Hospitals) and Orumieh (Imam Khomeini Hospital), Iran, between 2008 and 2010. The origins of isolates were as follows: urine 35 (72.91%), blood 5 (10.41%), wound 3(6.25%), body fluids 4(8.33%) and intravenous catheter 1(2.08%). Initially, these isolates were identified to the species level by conventional methods described previously [12] and then, multiplex PCR was used to detect vancomycin resistant *vanA* and *vanB* genes and also to confirm phenotypic speciation by targeting D-alanine– D-alanine ligases specific for *E. faecalis* ($ddl_{E.\ faecalis}$) and *E. faecium* ($ddl_{E.\ faecium}$) as developed by Kariyama *et al.*, [13]. *E. faecalis* E206 ($vanA^+$) and *E. faecium* E2781 ($vanB^+$) (kindly provided by Dr. Eimaneini) was used as control strains.

For all isolates, MICs of vancomycin were determined by agar dilution method and E-test (BioMerieux, SA) according to the CLSI's (2006) guidelines [14] and manufacturer's recommendations, respectively. *E. faecalis* ATCC 29212 was used as a quality control strain on every occasion of susceptibility testing.

Detection of Virulence Genes by PCR

DNA extraction was performed by boiling method as described previously [15] and then by commercial kit (DNG^{TM}-Plus, CinnaGen, Iran). Multiplex PCR was performed on all enterococcal isolates for the detection of virulence determinants with specific primers for each gene (Table **1**) with some modification on Vankerckhoven's protocol [16]. Briefly, first 25µl master mix containing 2.5µl of bacterial suspension, 15pM of each primers for *asa1* and *gelE* and 30 pM for *esp*, 1X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP,

Table 1. Primers used in this Study

Genes	Sequence (5' to 3')	Size(bp)	References
asa1	F1: GCACGCTATTACGAACTATGA R1: TAAGAAAGAACATCACCACGA	375	[15]
gelE	F2: TATGACAATGCTTTTTGGGAT R2: AGATGCACCCGAAATAATATA	213	[15]
esp	F7: AGATTTCATCTTTGATTCTTGG R7: AATTGATTCTTTAGCATCTGG	510	[15]
cpd	F8: TGGTGGGTTATTTTTCAATTC R8: TACGGCTCTGGCTTACTA	782	[29]
ace	F9: GGAATGACCGAGAACGATGGC R9: GCTTGATGTTGGCCTGCTTCCG	616	[14]
vanA	F14-CATGAATAGAATAAAAGTTGCAATA R14-CCCCTTTAACGCTAATACGATCAA	1030	[12]
vanB	F15-GTGACAAACCGGAGGCGAGGA R15-CCGCCATCCTCCTGCAAAAAA	433	[12]
E. faecalis	F16-ATCAAGTACAGTTAGTCTTTATTAG R16-ACGATTCAAAGCTAACTGAATCAGT	941	[12]
E. faecium	F17-TTGAGGCAGACCAGATTGACG R17-TATGACAGCGACTCCGATTCC	658	[12]

dGTP, and dTTP) and 2.5 U of *Taq* DNA polymerase (CinnaGen, Iran). The second PCR mixture contained 10 pM of each primer for *cpd* and 4pM for *ace*, 1.5 mM $MgCl_2$ and additional rest of reagents mentioned in first master mix. Reactions for both mixtures were done on a thermal cycler (ASTEC-Japan) with an initial denaturation at 95°C for 10 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min), and a final extension at 72°C for 10 min.

Amplicons were analyzed on a 1.5% agarose gel and a 100-bp DNA ladder was used as the molecular size marker. The gels were stained with ethidium bromide and photographed under UV light. Each PCR assay was accompanied with a negative control, containing all of the reagents without template DNA.

Statistical Analysis

Data were analyzed statistically using chi-square test and difference was considered significant at $p<0.05$ by SPSS software (version 18).

RESULTS

Most of VREs were isolated from intensive care units (ICUs) [16(33.3%)], nephrology [10(20.8%)] and internal [8(16.7%)] wards. Among VRE strains, thirty- five *E. faecium* harboured *vanA* gene and all of them revealed MICs≥ 256 µg/ml, except one (MIC=8µg/ml). Five *vanA* positive isolates did not show any studied virulence determinants. On the other hand, 3 *E. faecium* isolates carried *vanB* gene with MICs< 4 µg/ml and also, they were negative for all virulence determinants tested. However, VREfm isolates were found with the *esp* gene (71.05%).

All *E. faecalis* showed MICs ≥ 256 µg/ml and 8 isolates carried *vanA* gene while, in the two strains, neither *vanA* nor *vanB* gene were detected.

In comparison to *E. faecium*, vancomycin resistant *E. faecalis* strains were furnished with diverse combinations of virulence genes include: 4 strains presenting *asa1, gelE, ace* and *cpd*; 4 strains with *asa1, gelE and cpd*; 1 strain showed *gelE, ace* and *cpd* and another one displayed *gelE* and *cpd*. Among virulence genes studied, concomitant occurrence of *gelE* and *cpd* in all *E. faecalis* isolates was significant ($p<0.001$). Distribution of virulence genes among vancomycin resistant *E. faecalis* and *E. faecium* are shown in Table **2**.

Table 2. Distribution of Virulence Genes among Vancomycin Resistant *E. faecalis* and *E. faecium* Isolates

Virulence Genes	Vancomycin Resistant		Total (N=48)
	E. faecalis (N=10)	***E. faecium*** (N=38)	
gelE	10 (100)	3(7.89)	13(27.08)
asa1	8 (80)	3(7.89)	11(22.91)
cpd	10(100)	1(2.63)	11(22.91)
ace	5(50)	1(2.63)	6(12.5)
esp	0(0)	27(71.05)	27(56.25)

DISCUSSION

This study investigated prevalence of virulence factors in vancomycin resistant enterococci. The majority of VREs were *E. faecium* (38 out of 48 VREs). Similar enhancement in the prevalence of VRE, especially *E. faecium* has been noticed earlier in several studies from different countries [17-19]. This increase has been attributed mainly to the occurrence and spread of *vanA* and *vanB* positive VRE, which exhibited some virulence factors such as Esp (*esp*), cytolysin (*cyl*), hyaluronidase (*hyl*) [10]. In comparison among various sources of *Enterococci*, the same as clinical samples, farm animals, water and food products, isolates from clinical specimens have been attributed to have the highest virulence factors [20, 21]. In this study, *E. faecalis* and *E. faecium* strains show significantly different patterns in the incidence of virulence determinants. Similar to study of Eaton *et al.*, (2001), in our study, all *E. faecalis* strains harbored multiple virulence determinants.

The aggregation substance is a pheromone-inducible surface protein encoded by *asa1* gene involved in adherence to eukaryotic cell [22], which may increase the hydrophobicity of the enterococcal cell surface that may delay or prevent fusion with lysosomal vesicles [23], cell aggregation and conjugation [24]. Various reports are available for the prevalence of *asa1* in enterococcal isolates obtained from different sources. In this study 8 out of 10 *E. faecalis* harbored *asa1* gene and majority (62.5%) of them were being isolated from urine specimen. Waar *et al.*, (2002) reported high prevalence of *asa1* in *E. faecalis* isolates from liver transplant and septicemic patients and assumed that *asa1* might be associated with infection [25]. Baldassarri *et al.*, [26] indicated presence of this gene in seven out of 11 *E. faecalis* from endocarditis, with only one strain being *vanA* positive. The present study also revealed this gene in 3 (7.89 %) VREfm isolates from UTI, whereas other studies did not find *asa1* in *E. faecium* [16, 27, 28]. A much higher incidence (13%) has been reported by Elsner *et al.*, [29] in clinical *E. faecium* isolated from blood culture. A study on food and medical isolates of enterococci showed the *asa1* gene was always associated with the presence of pheromone determinants (*cpd, cob, ccf* and *cad*) and only detected in *E. faecalis* [30]. Abriouel *et al.*, (2008) reported high prevalence of *cpd* (89.7%) determinant in comparison of aggregation substance (58.97%) in clinical *E. faecalis* [28]. Similarly, we found the *cpd* gene in all VREfs, of them 80% were also *asa1* positive ($p<0.001$). Meanwhile, we could find *cpd* in one VREfm, suggests that it is probably not associated with virulence in this species. The presence of sex pheromone determinant in *E. faecium* was reported earlier [31].

Gelatinase encoded by *gelE* is an extracellular metalloendopeptidase that hydrolyzes gelatin, collagen, hemoglobin, and other bioactive compounds [32]. Sabia *et al.*, (2008) reported *gelE* gene in 19 (70%) out of 27 VRE strains; all *E. faecalis* and 5 out of 7 *E. faecium* carried the *gelE* [33]. In accordance, we detected *gelE* gene in all VREfs and 7.9% of VREfm. In fact, among virulence genes investigated, concomitant occurrence of *gelE* and *cpd* in *E. faecalis* isolates was found significant ($p<0.001$). In contrast, some studies did not find *gelE* gene in any *E.*

faecium isolates [34] while, other researchers reported different incidence for mentioned gene in clinical isolates [11, 27]. Silent form of this gene was reported in *E. faecium* isolates [30, 35].

The *ace* gene, which codes for a putative protein with characteristics similar to a collagen-binding protein of *Staphylococcus aureus* [36] has been identified more frequently in *E. faecalis* isolates [27, 37]. Abriouel *et al.*, (2008) reported higher incidence of *ace* gene (>80%) in clinical isolates in comparison to much lower incidence or even absence in isolates obtained from vegetable foods, water and soil [28]. The present study revealed the *ace* gene in 50% of *E. faecalis* and only in one *E. faecium*. All *ace* positive isolates obtained from urine (UTI) and isolates from other sources were negative for this gene. Lebreton *et al.*, (2009) suggested that Ace may be a valuable drug target against human UTI. Low incidence or absence of the *ace* in clinical *E. faecium* have been reported in some studies [11, 27] but it is unlikely to contribute significantly to virulence.

Esp protein encoded by *esp* gene assumed to play a role in the primary surface attachment, contributing to colonization and persistence on urinary tract and biofilm formation [38, 39]. The *esp* is one of the disputed virulence elements that found on a pathogenicity island of both *E. faecalis* and *E. faecium* [40, 41].

Although, an earlier study demonstrated *esp* gene only in *E. faecalis* isolates [42] and other available data indicated that the *esp* gene was common in *E. faecalis* [15] however, a study on the food and medical isolates depicted an increasing incidence of *esp* in clinical *E. faecium* isolates than *E. faecalis* [30] and an European investigation proposed, proposed only this gene for virulence among this species [16]. Camargo *et al.*, (2006) demonstrated that *esp* (56%) was restricted to VREfm and not found in vancomycin sensitive *E. faecium* [43]. Vankerckhoven *et al*., [16] surveyed virulence genes in European hospitals and found higher incidence of *esp* in the clinical VREfm isolates. In accordance with these investigations, in our study *esp* was the most common gene in VREfm (71.05%) but not detected in VREfs. Moreover, earlier Van Wamel *et al*., (2007) demonstrated that Esp expression on the surface of *E. faecium*; dependent to growth condition, to vary consistently among strains and is quantitatively correlated with initial adherence and biofilm formation [44].

In the present study, three *E. faecium* isolates with *vanB* genotype were susceptible to vancomycin (MIC< 4 µg/ml) and did not carry any of examined virulence determinants. A research conducted on Australian patients with haematological disorders showed high incidence of *esp* gene in vancomycin resistant *E. faecium* (*vanB* positive), but all were negative for *asa1* and *gelE* [45].

CONCLUSION

Our data indicate a high prevalence of *E. faecium* harboring vancomycin resistance with *vanA* genotype. In addition, finding of 3 *E. faecium* with *vanB* genotype, but sensitive to vancomycin, suggest application of both phenotypic and genotypic methods for screening VRE strains since using them in single may lead to misidentification.

VREfs possessed four distinctive patterns of virulence factors and the *gelE* gene was always associated with the presence of pheromone determinant (*cpd*) and these combinations may associate with increased virulence. Interestingly, *esp* was not found in any of VREfs strains.

On the other hand, the *esp* was the dominant determinant among *E. faecium* strains. Finding of *esp* gene in high incidence among *E. faecium*, but not in *E. faecalis*, may indicate the role of this gene in high prevalence of VREfm in hospitals environments. Although the *gelE*, *cpd*, *asa1*and *ace* genes were found in less frequencies, but there is a trend of increasing levels of virulence genes other than *esp* among *E. faecium* strains.

Meanwhile, in 8 of VREfm (3 *vanB*$^+$ and five *vanA*$^+$) none of these genes was present, it was concluded that other genes may be also important in the pathogensis of VRE isolates.

However, further investigation is required to evaluate the association of these virulent determinants with occurrence of an infection by VREs in more number of isolates.

CONFLICT OF INTERESTS

Declared none.

ABBREVIATIONS

Ace = Collagen binding protein
Asa1 = Aggregation substance
ATCC = American type culture collection
CLSI = Cinical and Laboratory Standards Institute
Esp = Enterococcal surface protein
GelE = Gelatinase
MICs = Minimum inhibitory concentrations
Min = Minutes
VanA = D- Ala- D- Lac ligase
vanB = D- Ala- D- Lac ligase
VRE = Vancomycin resistant Enterococci
VREfm = Vancomycin resistant *Enterococcus faecium*
VREfs = Vancomycin resistant *Enterococcus faecalis*

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