JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Prevalence of virulence genes and their association with biofilm formation in VRE *faecium* isolates from Ahvaz, Iran

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Abstract

Introduction: Vancomycin-resistant *Enterococcus faecium* (VREfm) is a common cause of nosocomial infections. Biofilm formation is an important factor in recurrence of infections, facilitating transfer of genetic elements, leading to treatment failures. The aim of this study was to investigate the virulence genes in biofilm producing isolates and to determine possible association between biofilm formation and the presence of these genes; also to determine association between antibiotic susceptibility patterns of VREfm isolates and their biofilm formation ability.
Methodology: A total of 57 isolates of VREfm were recovered from different sources of hospitals under Ahvaz University, Iran. The isolates were examined by conventional microbiological methods and molecular test using PCR. The antibiotic susceptibility patterns of the isolates were determined by disk-diffusion and E-test. The biofilm formation ability of the isolates was investigated by Modified Congo red agar and microtiter plate techniques. The presence of virulence genes was examined using Multiplex-PCR method.
Results: Out of 57 VREfm isolates, 63.15% of isolates were biofilm producers. The frequency of biofilm producing isolates from clinical specimens, colonized patients and environmental sources were 78.26%, 60%, and 42.85%, respectively. The prevalence of *acm*, *esp* and *hyl* genes among biofilm producing isolates was 86.10%, 55.56% and 52.77%, respectively. There was statistically significant association between *esp* gene and biofilm formation among isolates from the clinical specimens.
Conclusion: Clinical isolates producing biofilms showed a positive association with the presence of the *esp*. Our study further suggests that the link between virulence genes and biofilms is affected by the environmental context.

Key words: *Enterococcus faecium,* VRE, biofilms, different source, virulence gene.

J Infect Dev Ctries 2018; 12(11):970-977. doi:10.3855/jidc.10078

(Received 16 December 2017 – Accepted 09 September 2018)

Introduction

Vancomycin-resistant enterococci are among the most common cause of nosocomial infection including urinary tract infections, wound infections, sepsis, bacteremia, meningitis and endocarditis [1]. The treatment of these infections is limited due to antibiotic resistance of this bacterium and its ability to adapt hostile hospital environment and to acquire more resistance mechanisms [2-4]. Eradication of *Enterococcus faecium* with antibiotic is very difficult when the bacterium develops persistent biofilm.

The expression and effect of virulence factors of this pathogen, such as enterococcal surface protein (Esp) and other different cell surface proteins, are usually related to its adhesion capability [5-6]. Genetic analysis confirmed that collagen-binding adhesin (Acm) is another virulence factor of *E. faecium* that is essential in mediating attachment of *E. faecium* strains to collagen [7]. Also *hyl* gene encodes a putative glycoside hydrolase which seems to facilitate intestinal colonization and peritoneal invasion [8]. The ability to form biofilm by *E. faecium* is strongly related to specific conditions such as urinary tract infections [9,10]. The *esp* gene, encodes Esp protein, likely has a role in colonization and persistence of *E. faecium* and probably interacts with primary surfaces and engages in biofilm formation [11]. This gene is seen frequently in isolates related to infections and hospital outbreaks [12-14] and the expression of Esp correlates with initial adherence to biofilm formation [15]. Nonetheless, so far, the conclusive role of Esp in biofilm formation could not be determined [16]. An important virulence

property of *E. faecium* is its ability to form biofilm on abiotic surfaces [17,18]. The Structure of biofilm creates suitable environment for interchange of genetic materials among bacteria and reinforces the innate resistance to antibiotic and triggers off the host immune response [18,19].

Antibiotic resistance has become a serious threat to clinicians for decades. Vancomycin resistant enterococci such as *E. faecium* are important emerging pathogens in nosocomial infections and have been cultured from environmental surfaces and from equipment of healthcare workers in hospital settings [20]. The most prevalent form of enterococcal antibiotic resistance is multidrug in nature [21].

Accurate knowledge of virulence factors of *E. faecium* is vital to understanding pathogenic process of this opportunistic pathogen [22]. Early diagnosis infections with biofilm producing *E. faecium* should be an essential component of infection prevention strategy of any hospital. Also determination of appropriate antibiotic susceptibility pattern helps physicians in prescribing appropriate drug, reducing the cost of treatment and hospitalization. Lack of comprehensive research on biofilm producing Enterococcus isolates in Ahvaz University Hospitals, Iran, forms the basis of this study. The aim of the study was to investigate the presence of *esp, hyl* and *acm* genes in biofilm producing Enterococcus isolates and to determine possible association between biofilm formation and the presence of these genes. Also to determine association between antibiotic susceptibility patterns of vancomycin resistant *E. faecium* isolates recovered from specimens associated with infection, colonization and hospital environment with their biofilm formation ability.

Methodology

Isolates collection and Identification

The study was carried on specimens recovered from in-patients (clinical samples), out-patients (colonization) and environment of hospitals under Ahvaz University, Iran, between February and July, 2016. A total of 57 isolates confirmed to be VREfm were recovered from clinical specimens associated with infections (n = 23), colonization (n = 20) and the environment (n = 14) of hospitals.

For confirmation, the received isolates were examined for positive Gram's reaction and biochemical assay including negative catalase test, the ability to grow on Bile-esculin-azide agar, ability to growth in the presence of 6.5% NaCl, the positive pyrrolidonyl arylamidase (PYR) test, and fermentation of 1% (w/v) of some sugars, such as sucrose, sorbose, sorbitol, L-arabinose, D-ribose, raffinose, arginine, were conducted [22,23]. *vanA*, *vanB* and *E. faecium ddl* genes (using primers Table 1) [24-26] were tracked using PCR and vancomycin resistance as well as genotypic identification of the isolates were confirmed up to species level.

Resistance to antibiotic tests

Antimicrobial susceptibility of the *E. faecium* isolates to ten antimicrobial agents (MAST Diagnostics, UK) including Ampicillin (10 µg), Teicoplanin (30µg), Vancomycin (30 µg), High Level Gentamicin, HLG (120µg), Chloramphenicol (30 µg) Nitrofurantoin (300 µg), Linezolid (30 µg), Erythromycin (15 µg), Quinopristin-Dalfopristin (15 µg) and Ciprofloxacin (5 µg), was determined by Kirby-Bauer disk diffusion method. Minimum inhibitory concentration (MIC) of Vancomycin and Teicoplanin were determined using Epsilometer test (E-test). *E. faecalis* ATCC 29212 was used as the quality

Table 1. Target genes and primers for identification of ddl, vanA, vanB, esp, hyl and acm genes.

Target Genes	Primer name	Sequence(5´-3´)	Size(bp)	References
ddl	*ddl E. faecium-F*	TTGAGGCAGACCAGATTGACG	658	[24]
	ddl E. faecium-R	TATGACAGCGACTCCGATTCC		
vanA	*vanA-F*	CATGAATAGAATAAAAGTTGCAATA	1030	[24]
	vanB-R	CCCCTTTAACGCTAATACGATCAA		
vanB	*vanB-F*	GTGACAAACCGGAGGCGAGGA	433	[24]
	vanB-R	CCGCCATCCTCCTGCAAAAAA		
esp	*esp-F*	AGATTTCATCTTTGATTCTTGG	510	[25]
	esp-R	AATTGATTCTTTAGCATCTGG		
hyl	*hyl-F*	ACAGAAGAGCTGCAGGAAATG	276	[25]
	hyl-R	GACTGACGTCCAAGTTTCCAA		
acm	*acm-F*	GGCCAGAAACGTAACCGATA	135	[26]
	acm-R	AACCAGAAGCTGGCTTTGTC		

control strain. The results were interpreted based on Clinical and Laboratory Standards Institute (CLSI) Guidelines-2016 [27].

Phenotypic study of biofilm formation
Modified Congo red agar method

All the *E. faecium* isolates were cultured on Modified Congo Red Agar (MCRA) plates to study phenotypic production of biofilm as described previously [28,29]. Congo red agar plate (CRA) made by mixing 0.8g of Congo red dye (Merck, Darmstadt, Germany) with 36g of saccharose (Sigma, St. Louis, USA) in one liter of Brain Heart Infusion Agar (BHI agar, Merck, Darmstadt, Germany) and incubated for 24 hours at 37 °C, and maintained over night at room temperature. The morphology of colonies was interpreted based on colonies colors including red, almost black, black, and very black. Red colonies considered as strains unable to produce the biofilm and weak biofilm production activity was inferred from almost black color. While very black and black colonies were regarded as strong biofilm producers.

Microtiter plate assay

The quantitative biofilm production was determined by modified Microtiter plate assay as previously explained [29,30]. Briefly, the bacterial suspension grown in Trypticase-Soy broth (Merck, Darmstadt, Germany) supplemented with 0.5% glucose and incubated at 37 °C overnight. Fresh TSB, supplemented with 0.5% glucose was used to dilute the cultures in 1:40. Then 200 µL of the diluted solution was added to wells of a microtiter plate and incubated for 48 hours at 37 °C. The negative control wells contained TSB supplemented with 0.5% glucose. Then, the cells were drawn off, and each well was carefully washed 3-times with sterile phosphate buffered saline (PBS; pH 7.2). Then methanol was used for 20 minutes and dried at room temperature, and the end stained with 0.1% safranin. The safranin dye bound to the adherent cells was dissolved with 200 µL of 95% ethanol per well. Then, optical density (OD) of wells was determined at 490 nm (490A) by ELISA reader. Optical density cut-off (ODc) defined as average OD of negative control + 3 × standard deviation (SD) of negative control. Finally, average OD value of negative controls and samples was calculated and interpretation of results was described as follow in Table 2. Formation of biofilm by isolates was analyzed and categorized based on the absorbance of the safranin-stained attached cells. Biofilm-producing *Staphylococcus epidermidis* ATCC 35984 was used for the biofilm producing control strain.

The study of virulence gene by Multiplex-PCR

The genomic DNAs of *E. faecium* isolates were extracted by boiling method [31]. The *acm, esp* and *hyl* genes were studied by Multiplex-PCR. PCR amplification was carried out using amplification parameter as follows: an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 1 minute, followed by an additional extension step of 10 minutes at 72 °C. The amplified products were electrophoresed on 1% agarose gel containing 1x GelRed DNA stain (Biotium, Inc., USA).

Statistical analysis

All obtained data such as relation between the antibiotic resistance, biofilm formation ability and presence of the genes among *E. faecium* isolates were analyzed by the Pearson Chi-Square test using SPPS software version 22 and P values less than 0.05 were considered as significant.

Results

Identification of species

A total of 57 isolates was recovered from all the three sources and were confirmed to be *E. faecium*.

Antibiotic susceptibility testing

The result showed different levels of resistance of the total isolates to the tested antibiotics follows: vancomycin (100%), teicoplanin (100%), ampicillin (94.73%) erythromycin (96.49%), gentamycin

Table 2. Classification and biofilm formation ability of isolates using microtiter palates method.

Cut-off value calculation	Mean of OD values results	Biofilm formation abilities
OD > 4 × ODc	OD > 0.242	Strong
2 × ODc < OD ≤ 4 × ODc	0.121 < OD ≤ 0.242	Moderate
ODc < OD ≤ 2 × ODc	0.060 < OD ≤ 0.121	Weak
OD ≤ 0.060	OD ≤ 0.060	None

(92.98%) quinopristin-dalfopristin (50.87%), nitrofurantoin (45.61%), chloramphenicol (22.80%) and ciprofloxacin (88.47%), while nil resistance was recorded to linezolid (Figure 1).

Biofilm production

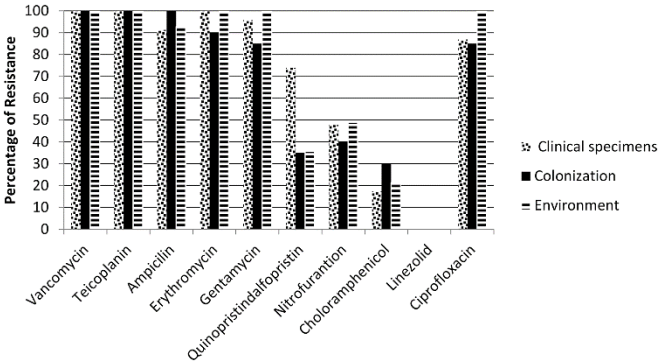
Biofilm formation of *E. faecium* was evaluated using Modified Congo red agar (MRCA) Method as well as using Microtitre plate method. Out of 57 VREfm isolates, 63.15% of isolates were biofilm producers by MRCA. The frequency of isolates producing biofilm from clinical specimens, colonized patients and environment was 78.26%, 60%, and 42.85% respectively. Meanwhile, in Microtitre plate method, among *E. faecium* isolates from all sources 63.15% of them were biofilm producers. There were differential levels of biofilm formation with regards to different sources of the isolates. The lowest percentage of strong adherent was seen in environmental source (7.14%), and the highest percentage of strong adherent was observed in intestine-colonized patients (20%). Moderate adherent was most seen in the infectious patients. The highest percentage of weak adherent was observed in infectious patients and the lowest percentage of weak adherent was produced in environmental sources (Table 3).

Table 4 shows the relationship between antibiotic resistance patterns of the isolates and biofilm formation using microtiter plate method. The results showed that there were significant differences between biofilm formation of VREfm isolates associated with colonization and resistance to some antibiotics.

Multiplex-PCR detection of virulence genes

The prevalence of *acm*, *esp* and *hyl* genes among clinical, colonization and environmental isolates was 82.45%, 50.87 % and 35%, respectively. The prevalence of *acm*, *esp* and *hyl* genes among biofilm producer isolates was 86.10%, 55.56% and 52.77%, respectively. Among the clinical isolates, 73.90%, 60.87%, and 34.78% had *acm, esp* and *hyl* genes, respectively. While isolates associated with colonization had 95%, 55% and 30%, of *acm, hyl* and *esp* genes, respectively and finally, isolates from the environmental source had 78.58%, 64.28% and 7.14% present *acm, esp* and *hyl* genes, respectively. Statistically significant association between *esp* gene and biofilm formation (p = 0.021) was seen among isolates from clinical specimens alone (Table 5).

Figure 1. Antimicrobial resistance pattern of E. faecium from different sources.



Discussion

E. faecium is one of the common causes of nosocomial infections. One important step in infection process of *E. faecium* is the attachment of bacteria to surfaces and biofilm formation. The formation of biofilm has an important role in transmission of genetic elements, antibiotic resistance and recurrence of infection. Due to high prevalence of antibiotic resistance among *E. faecium* isolates, there is urgent need to device other means of fighting this pathogen by halting biofilm formation or impeding the action of other virulence factors.

Table 3. Biofilm formation ability of E. faecium in Microtitre plate and Congo red agar methods (n = 57).

Method	Biofilm formation	Clinical isolates N = 23(%)*	Isolates from colonization N = 20(%)	Isolates from environment N = 14(%)	Total N = 57(%)
Modified Congo red agar	Very black	3(13.04)	3(15)	1(7.14)	7(12.28)
	Black	9(39.13)	6(30)	3(21.42)	18(31.58)
	Almost Black	6(26.08)	3(15)	2(14.28)	11(19.3)
	Red	5(21.74)	8(40)	8(57.14)	21(36.84)
Microtiter plate assay	Strong	4(17.39)	4(20)	1(7.14)	9(15.79)
	Moderate	9(39.13)	6(30)	4(28.57)	19(33.33)
	Weak	5(21.74)	2(10)	1(7.14)	8(14.03)
	None	5(21.74)	8(40)	8(57.14)	21(36.84)

*: urine, wound swab, blood etc.

Table 4. Biofilm formation and antibiotic resistance pattern of E. faecium from different sources (n = 57).

Antibiotic	Clinical isolates N(%)						p-value	Isolates from colonization N(%)						P-value	Isolates from environment N(%)						P-value
	R		I		S			R		I		S			R		I		S		
	+	-	+	-	+	-		+	-	+	-	+	-		+	-	+	-	+	-	
Ampicillin	16(69.6)	5(21.7)	0	0	2(8.7)	0(0)	0.672	12(60)	8(40)	0	0	0	0	0.0001	5(35.7)	8(57.1)	0	0	1(7.1)	0(0)	0.294
Teicoplanin	18(78.3)	5(21.7)	0	0	0	0	-	12(60)	8(40)	0	0	0	0	-	6(42.9)	8(57.1)	0	0	0	0	-
Nitrofurantoin	8(34.8)	3(13)	0	0	10(43.48)	2(8.7)	.354	3 (15)	5(25)	1(5)	0	8(40)	3(15)	3.818	4(27.6)	3(21.4)	0	0	2(14.3)	5(35.8)	0.424
Linezolid	0	0	0	0	18(78.3)	5(35.7)	-	0	0	0	0	12(60)	8(40)	-	0	0	0	0	6(42.9)	8(57.1)	-
Erythromycin	18(78.3)	5(21.7)	0	0	0	0	-	10(50)	8(40)	0	0	2(10)	0	-	6(42.9)	8(57.1)	0	0	0	0	-
Quinopristin-Dalfopristin	13(56.5)	4(17.4)	0	0	5(21.7)	1(4.3)	0.059	6(30)	1(5)	0	0	6(30)	7(35)	1.978	2(14.3)	3(21.4)	0	0	4(28.6)	5(35.7)	0.141
Ciprofloxacin	15(65.2)	5(21.7)	0	0	3(13)	0	0.672	9(45)	8(40)	3(15)	0	0	0	2.222	6(42.9)	8(57.1)	0	0	0	0	-
Gentamycin	18(78.3)	4(17.4)	0	0	0	1(4.3)	1.626	9(45)	8(40)	0	0	3(15)	0	2.222	6(42.9)	8(57.1)	0	0	0	0	-
Chloramphenicol	3(13)	1(4.3)	2(8.7)	0	13(56.5)	4(17.4)	1.662	4(20)	2(10)	0	0	8(40)	6(30)	0.000	0	3(21.4)	0	0	6(42.8)	5(35.7)	0.923
Vancomycin	18(78.3)	5(21.7)	0	0	0	0	-	12(60)	8(40)	0	0	0	0	-	6(42.9)	8(57.1)	0	0	0	0	-

R: Resistance; I: Intermediate; S: Susceptible; +: Biofilm positive; -: Biofilm Negative.

Table 5. Association between biofilm formation and presence of esp, acm and hyl genes among E. faecium from different.

Genes		Clinical isolates N(%)		Total N = 23(%)	P-value	Isolates from colonization N(%)		Total N = 20(%)	P-value	Isolates from environment N(%)		Total N = 14(%)	P-value
		Biofilm +	Biofilm -			Biofilm+	Biofilm-			Biofilm +	Biofilm-		
esp	Positive	11(78.60)	3(21.40)	14(60.87)	0.021	4(66.67)	2(33.33)	6(30)	0.386	5(55.55)	4(44.44)	9(64.28)	0.332
	Negative	7(55.50)	2(44.50)	9(39.13)		8(57.14)	6(42.86)	14(70)		1(20)	4(80)	5(35.72)	
acm	Positive	13(76.47)	4(23.53)	17(73.90)	0.436	12(63.15)	7(36.85)	19(95)	1.353	6(54.54)	5(45.46)	11(78.58)	3.871
	Negative	5(83.33)	1(16.67)	6(26.10)		0(0)	1(100)	1(5)		0(0)	3(100)	3(21.42)	
hyl	Positive	7(87.50)	1(12.50)	8(34.78)	0.441	11(100)	0(0)	11(55)	0.521	1(100)	0(0)	1(7.14)	1.221
	Negative	11(73.33)	4(26.67)	15(65.22)		1(11.20)	8(88.80)	9(45)		5(38.46)	8(61.53)	13(92.86)	

Microtitre plate technique is regarded as more accurate and more reliable method for investigating the ability of *E. faecium* to generate biofilm [29]. In our study, high percentage (63.15%) of *E. faecium* isolates produced biofilm. Similar study recently reported 75% of *E. faecium* isolates were biofilm producers [29]. Our results showed difference in the ability to form biofilm and respective source of isolates. The frequency of biofilm formation is higher among isolates recovered from specimens associated with infection and colonization than that recovered from environmental sources. This variation in range of biofilm production can be attributable to difference in the original source of the isolates. Since the *E. faecium* is one of nosocomial pathogens, identification of its virulence factors is very important. In this study, the distribution of virulence genes (*esp*, *hyl* and *acm)* was investigated among isolates from all three sources.

Because the biofilm formation may be a multifactorial process, the exact link in the association between enterococcus virulence factors and biofilm formation is difficult to determine [32]. There are conflicting results about the role of virulence genes including *esp* in biofilm formation [33-35]. Some studies suggested that a single gene was not sufficient for biofilm formation, and environmental factors and genetic factors may be associated with the production of biofilm [29,36]. Other factors, such as nutrient concentration of media, can affect biofilm formation of *E. faecium* [37,38]. Though previous studies reported the role *esp* gene in biofilm formation among *E. faecalis* [39-42] and another study [11] found that *esp* gene is not associated with heavy biofilm formation in *E. faecium*, the result of our study indicated that *esp* gene might have significant role to play in biofilm formation among clinical patients. Our results seem to confirm that from other studies [43].

In our study antibiotic susceptibility patterns of *E. faecium* from different sources was evaluated. We observed different patterns of antibiotic resistance among isolates from different source of *E. faecium*. Multiple drug resistance (MDR) was seen in all studied isolates. This multiresistance attribute is a common feature and frequently being observed among isolates of *E. faecium* [44,45]. High resistance was recorded to vancomycin, teicoplanin, ampicillin, erythromycin, gentamycin and ciprofloxacin. Although, the strains were also susceptible to nitrofurantoin and chloramphenicol, linezolid was recorded as the most effective antibiotic against *E. faecium* strains. This result is in accordance with recently reported findings that linezolid, chloramphenicol and nitrofurantoin were the most effective agents against enterococcal isolates [29]. On the other hand, the lowest amount of biofilm production was observed despite presence of virulence genes among isolates from environmental source. This may be related to the proposed multifactorial requirements for biofilm production by the pathogen.

Conclusion

The highest biofilm production was seen among clinical isolates. The high prevalence of *esp* gene found among biofilm-producing clinical isolates suggests potential link between biofilm formation and the *esp* gene, especially among clinical patients; though other virulent genes with potentials connection to biofilm formation such as pili genes should have been studied. An *esp* positive isolates showed highest antibiotic resistance. Linezolid remains the most effective agent against the clinical isolates.

Acknowledgements

This research was supported by Tehran University of Medical Sciences, Tehran, Iran (grant number: 35110)

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Conflict of interests: No conflict of interests is declared.