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Prevalence of vancomycin-resistant enterococci from three tertiary care hospitals and role of *esp* gene in biofilm formation

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Abstract

The purpose of this study was to elucidate antibiotic resistance profile of 143 *Enterococcus* spp. isolates against vancomycin and other antimicrobial agents from three Pakistani hospitals and to show a relationship between biofilm formation and *esp* gene as virulence factors. Different specimens, mainly, urine, blood and pus were processed for this purpose. Antibiotic resistance profile was elucidated through Kirby-Bauer disc diffusion and E-test methods. Isolates were screened for biofilm formation using microtiter plate method and the presence of *esp* gene was determined through Polymerase Chain Reaction (PCR). A Chi-square (χ^2) test was used to find association between biofilm formation and the presence of *esp* gene. Among 143 isolates, 25 (17.48%) were found to be Vancomycin-Resistant Enterococci (VRE). The resistance against linezolid was very low, i.e., only 01 isolate was resistant and 07 intermediately resistant. None of the isolates were found resistant to tigecycline. Among VRE, 90.5% *Enterococcus faecium* and 100% *Enterococcus faecalis* isolates were positive for biofilm formation. The association between biofilm formation and *esp* gene was found insignificant (p-value >0.05) for both vancomycin-resistant and sensitive isolates. The frequency of vancomycin resistance in *Enterococcus* spp. is gradually increasing in Pakistan. Beyond vancomycin, tigecycline, linezolid and teicoplanin were the most effective antimicrobial agents in a local hospital setting. Biofilm was formed in most of the isolates, which might cause nosocomial infections, regardless of the presence of *esp* gene.

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Introduction

Bacterial resistance to antimicrobial agents has tremendously increased over the past few years, all over the world (Livermore, 2009). *Enterococcus* and *Staphylococcus aureus* are among the most protuberant gram-positive organisms with the remarkable potential of carrying resistance and clinical impact (Theuretzbacher, 2013). Till date, 44 species of *Enterococcus* have been described, with *Enterococcus faecium* and *Enterococcus faecalis,* the most common infectious agents in humans (Gilmore *et al*., 2013). Enterococci have a complex reservoir for resistance, which interacts with humans, animals, from livestock to wild animals and even to migrating birds where the exchange of resistance genes occurs efficiently from diverse gene pools (Hammerum, 2012).

Enterococci are recognized as the second and third most common cause of urinary tract infections and nosocomial bacteremia, respectively (Gaspar *et al*., 2009, Lindenstrauss*et al*., 2011). They emerge as challenging pathogens because of increasing antibiotic resistance and due to the acquisition of various virulence factors. The most commonly encountered species are *E. faecalis* and *E. faecium* but the proportion of *E. faecium* is increasing over time (Murdoch *et al*., 2002, Treitman *et al*., 2005).

Enterococci have become resistant to many antimicrobial agents including vancomycin. VRE infections are frequently appearing as hospital outbreaks that present great challenges for infection control surveillances with minimum treatment options (Rubinstein and Keynan, 2013), and contributing to substantial morbidity and mortality (De Kraker *et al*., 2011). VRE are a common cause of urinary tract infections (UTIs) and have been associated with wound infections, septicemia, peritonitis, endocarditis and meningitis (da Silva *et al*., 2014). Tigecycline is an effective, last resort antibiotic against many bacterial populations including *Enterococcus* spp. (Olson *et al*., 2006, Waites *et al*., 2006).

Virulence of an organism is regulated by virulence coding genes present in special regions of the genome which are termed Pathogenicity Islands (PAIs) (Upadhyaya *et al*., 2009). Enterococcal virulence is associated with different factors, such as aggregation substance (AS), enterococcal surface protein (*esp*), gelatinase production, and biofilm formation (Chuang *et al*., 2009).

The enterococcal surface protein, encoded by the *esp* gene, acts as an adhesin in the colonization and persistence of *E. faecalis* strains in ascending infections of the urinary tract. In addition, there is experimental evidence that *esp* may mediate the interaction with primary surfaces and participate in biofilm formation (Shankar *et al*., 2001, Chuang-Smith *et al*., 2010). Biofilm substantially enhances survival of bacteria in biopolymers which helps the organisms colonize and cause infection (Upadhyaya *et al*., 2010).

VRE prevalence is reportedly increasing gradually for last several years. Virulence factors in enterococci from clinical settings have rarely been reported in Pakistan, although there is a gradual increase in the prevalence of enterococcal infections. The purpose of the current study was to assess VRE frequency and any associationbe tween biofilm formation and presence of *esp* gene, in different types of infections in tertiary care hospitals of Pakistan. Besides this, the activity of other antibiotics against VRE strains from different specimens was also checked.

Materials and methods

Sampling

This prospective study was carried out at the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan. Clinical samples were collected from three major teaching hospitals of Islamabad [Pakistan Institute of Medical Sciences (P.I.M.S) & Polyclinic Hospital] and Rawalpindi (Holy Family Hospital) during the period from October 2013 to May 2014, after ethical approval granted by the concerned Ethical Committees. The sources of clinical specimens from indoor hospitalized patients were urine, blood, pus, tissue, catheter tips, high vaginal swab, and perirectal swab.

Identification of bacterial strains

Culture swabs were inoculated onto bile aesculin agar (BAA) (toxoid UK) plates and incubated for 24 hours at 37°C. Pinpoint colonies with black hallow around were inoculated on selective medium, Chromocult Enterococci Agar (Merck, Germany), where red colonies were given by enterococci only, while other bacteria gave blue, purple, colorless and turquoise colonies. Species identification was done by carbohydrates utilization test (*E. faecium* is positive to arabinose and raffinose fermentation test while *E. faecalis* is positive to sorbitol). Only *E. faecium* can grow at 4°C. All confirmed enterococci isolates were preserved in 30% v/v glycerol broth in cryovials at -80°C.

Screening of enterococcal isolates for vancomycin resistance

A selective medium supplemented with vancomycin, Chrom ID VRE (Biomerieux, France) was used for detection and differentiation of *E. faecium* and *E. faecalis* showing resistance to vancomycin.

Antimicrobial susceptibility testing

All the clinical isolates were screened for antibiotics susceptibility testing using the Kirby-Bauer modified disc diffusion method (Mulder *et al.*, 1995). A panel of antibiotics evaluated included: Vancomycin (VA, 30 µg), Linezolid (LNZ, 30 µg), Gentamycin (CN, 120 µg), Tigecycline (TGC, 15 µg), Teicoplanin (TEC, 30 µg), Norfloxacin (NOR, 10 µg), Rifampicin (RD, 5 µg), Ciprofloxacin (CIP, 5 µg), Ampicillin (AMP, 10 µg), Chloramphenicol (C, 30 µg) and Nitrofurantoin (F, 100 µg). *E. faecalis* ATCC 29212 and *E. faecium* ATCC 51858 were used as quality control strains. Results were interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines, 2013 (CLSI., 2013). Minimum inhibitory concentrations (MICs) was determined for VA, TGC, LNZ and TEC with Epsilometer test (E-test) (Oxoid, UK) method. Results for VA, LNZ and TEC were interpreted according to CLSI, but for TGC, British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2013) was followed.

Biofilm assay

Bacteria were grown overnight in *Trypticase soy broth (*TSB), diluted 1:100 in 200 µl of TSB with 0.25% glucose and inoculated onto flat bottom polystyrene microtiter plates (Falcon, Franklin Lakes, N.J.). After 24 hours of static incubation at 37°C, plates were gently washed, fixed with Bouin's fixative for 30 minutes, stained with 1% crystal violet (CV) for 30 minutes and rinsed with distilled water. CV was solubilized in ethanol-acetone (80:20, v/v), and optical density at 570 nm (OD^{570}) was determined. Reference strains *E. faecium* TX82 and *E. faecium* TX16 (DO) were used as positive and negative controls, respectively. Each assay was performed in quadruplicate on at least three occasions (Mohamed *et al.*, 2004).

PCR for the detection of esp gene

All isolates were tested for the presence of the *esp* gene by PCR. DNA was extracted using phenol-chloroform extraction. The *esp* gene amplification was performed using primers e*sp* F (5`-TTGCTAATGCTAGTCCACGACC-3`) and e*sp* R (5`GCGTCAACACTTGCATTGCCGAA -3`) as described previously (Shankar *et al.*, 1999). PCR conditions were following; initial denaturation at 95°C for 5 minutes, then underwent 35 cycles of denaturation at 95°C for 1 minute, annealing (58°C for 30 sec), extension (72°C for 30 seconds) and final extension (72°C for 10 min). Amplified PCR products were loaded on 1% agarose gel (stained with ethidium bromide) and gel electrophoresis was carried out for 35 minutes at 110 Voltage. Bands were observed under UV trans-illuminator, 1kb ladder (Invitrogen) was used as a size marker.

Statistical analysis

Statistical analysis of the data was produced with Minitab 16 software (State College, PA, USA). Pearson's chi-square test or the chi-square test of association, was used to find out if there is a relationship between two categorical variables, VRE and Vancomycin-Sensitive Enterococci (VSE) isolates with respect to the production of biofilm and *esp* gene.

Results

Collection of clinical isolates

Among 143 *enterococci* clinical isolates collected from hospitalized patients, 63.63% (n= 91) were *E. faecium* and 36.36% (n= 52) were *E. faecalis*.

Our main sample sources were Urine (n= 73), pus (n= 40), blood (n= 20), tissue (n= 03), catheter tips (n= 04), perirectal swab (n= 02) and HVS (high vaginal swab) (n= 01) (Table 1).

Table 1. Percentage of isolates on the basis of specimen type (n=143).

Specimens	Number of isolates	Percentage (%)
Urine	73	51
Pus	40	28
Blood	20	14
Catheter tips	04	2.8
Tissue	03	2
Perirectal swab	02	1.4
High vaginal swab	01	0.7

Antimicrobial disc susceptibility testing

Out of total 143 clinical isolates, 25 were found resistant to VA, followed by 72 AMP, 09 TEC, 11 LNZ, 103 CN, 119 CIP, 58 C, 3 TGC, 51 F, 120 RD and 116 NOR (Table 2).

Table 2. Susceptibility pattern of Enterococcus isolates collected from three tertiary care hospitals, against different groups of antibiotics.

Antibiotics	All Isolates n = 143 No. of isolates (%)	Isolates from PIMS n = 86 No. of isolates (%)	Isolates from HFH n = 35 No. of isolates (%)	Isolates from PCH n = 22 No. of isolates (%)
Gentamicin	103 (72)	63 (73.2)	23 (65.7)	17 (77.2)
Rifampicin	120 (83.9)	77 (89.5)	26 (74.2)	17 (77.2)
Ciprofloxacin	119 (83.2)	71 (82.5)	31 (88.5)	17 (77.2)
Norfloxacin	116 (81.1)	71 (82.5)	27 (77.1)	18 (81.8)
Vancomycin	25 (17.4)	16 (18.6)	06 (17.1)	03 (13.6)
Teicoplanin	09 (6.3)	05 (5.8)	03 (8.6)	01 (4.5)
Tigecycline	03 (2.0)	02 (2.3)	01 (2.8)	0 (0)
Linezolid	11 (7.6)	07 (8.1)	01 (2.8)	03 (13.6)
Ampicillin	72 (50.3)	44 (51.1)	15 (42.8)	13 (59)
Nitrofurantoin	51 (35.6)	23 (26.7)	15 (42.8)	13 (59)
Chloramphenicol	58 (40.5)	29 (33.7)	17 (48.5)	12 (54.5)

PIMS: Pakistan Institute of Medical Sciences, Islamabad.
HFH: Holy Family Hospital, Rawalpindi.
PCH: Poly Clinic Hospital, Islamabad.

Antibiotic resistance profile of clinical isolates showed a high rate of resistance to VA 17.48% (n=25), which included 12.58% (n=18) *E. faecium* and 4.89%, (n=07) *E. faecalis*. Among total VRE isolates, 100% were resistant to RD, AMP, CIP, NOR and CN, followed by F (n=19), C (n=17) and TEC (n=09).

Only one isolate each was resistant to LNZ and TGC. Maximum number of VRE were isolated from urine specimen (n=15), followed by blood (n=06) and perirectal swab (n=02) (Table 3).

Table 3. Frequency of vancomycin resistant enterococci in different clinical specimens (n=25).

Sample	Frequency	Percentage (%)
Urine	15	60
Blood	06	24
Pus	01	4
Tissue	0	0
Catheter tips	01	4
Perirectal swab	02	08
High vaginal swab	0	0

Measurement of MICs

Out of all VRE isolates, for VA (breakpoint (bp) ≥ 32 mg/L) 13 out of 25 had MIC ≥256 µg/mL, followed by 08 with 128 µg/mL, 03 with 32 µg/mL and 01 with ≥02 µg/mL, while with TEC (bp ≥ 32 mg/L) MIC for 09 out of 25 isolates ranged between 24-64 µg/mL. MIC for TGC (bp ≥ 0.5 mg/L) for 03 isolates was in the range of 0.25-0.42 µg/mL. Likewise, MIC for LNZ (bp ≥ 08 mg/L) for 11 isolates ranged from 0.5-8 µg/mL (Table 4).

Table 4. MIC values for different antibiotics against resistant enterococcal isolates.

Antibiotics/Resistant Isolates	MIC Value (µg/mL)	No. of Isolates (%)
Vancomycin (VRE isolates n=25)	≥256	13(52) R[a]
	≥128	08(32) R[a]
	≥32	03(12) R[a]
	≥02	01(04) S[c]
Teicoplanin (VRE isolates n=25)	64	06 (24) R[a]
	32	03 (12) R[a]
	24	16 (64) S[c]
Tigecycline (n=03)	0.42	01 (33) S[c]
	0.25	02 (67) S[c]
LNZ (n=11)	08	01 (09) R[a]
	04	07 (63.6) I[b]
	≤02	03 (27.2) S[c]

a = Resistant, b = Intermediate, c = Sensitive.

Biofilm formation

Among the 143 isolates, 33% were strong biofilm formers, 24% were moderate and 29% were weak, while 14% were non-biofilm formers. Among 92 *E. faecium* isolates, the maximum OD^{570} absorbance value for crystal violet stained biofilms was 1.3 (range 0.01-1.3). Taking range(0.2-1.3) for biofilm formation, 78.9% VSE *E. faecium* were positive biofilm formers while 90.5% VRE *E. faecium* were positive biofilm formers. Among *E. faecalis* isolates, the maximum OD^{570} absorbance value was 3.2 (0.3-3.2). Taking range (0.3-3.2) for biofilm formation, 93.6% VSE *E. faecalis* and 100% VRE *E. faecalis* isolates were positive biofilm formers (Table 5).

Association between biofilm formation and esp gene

PCR was performed for detection of *esp* gene in all 143 isolates. The amplified product of 920 bp was run on agarose gel and bands were observed.

Presence of *esp* gene in VRE and VSE *E. faecium* was found positive in 85.7% and 74.6% isolates, respectively.

Table A shows the biofilm formation and presence of the *esp* gene, as observed in VSE *E. faecium* (χ^2test: *p*-value = 0.592). Whereas Table B shows the biofilm formation and presence of the *esp* gene, as observed in VRE *E. faecium* (χ^2test: *p*-value = 0.368).

VRE and VSE *E. faecalis* were found 25 and 68% *esp* positive, respectively (Table 5). The biofilm formation and presence of the *esp* gene, in VSE *E. faecalis*is shown in table C (χ^2test: *p*-value = 0.592).

Table 5. Comparison among *E. faecium* and *E. faecalis* isolates for production of biofilm and *esp* gene.

Factors	*E. faecium* (n=92)		*E. faecalis* (n=51)	
	VSE (n = 71) (%)	VRE (n = 21) (%)	VSE (n = 47) (%)	VRE (n = 04) (%)
Biofilm	56 (78.9)	19 (90.5)	44 (93.6)	04 (100)
Esp gene	53 (74.6)	18 (85.7)	32 (68)	01 (25)

Chi-square (χ^2) test could not be computed for 4 VRE *E. faecalis* isolates. The *p*-value is greater than 0.05 and hence not significant, therefore the biofilm and *esp* gene were found independent from each other.

Further analysis of clinical isolates revealed that 79% and 96% *E. faecium* and *E. faecalis* isolated from urine specimens were biofilm-formers while the *esp* gene was present in 75% and 60% isolates, respectively. *E. faecium* isolates from specimens of blood (80%), pus (81.8%), catheter tips (66.7%) and perirectal swab (100%) showed biofilm production, while 93.3%, 68.1%, 100% and 100% *E. faecalis* isolates were found positive for the *esp* gene, respectively. Similarly, *E. faecalis* isolates from specimens of urine (96%), blood (83.3%), pus (88.8%), tissue (100%) and catheter tips (100%) showed biofilm formation, while 60%, 66.6%, 72.2%, 50% and 0% *E. faecalis* isolates were found positive for the *esp* gene, respectively (Table 6).

Table 6. Comparison between biofilm formation and *esp*gene among different clinical specimens.

Clinical samples	Biofilm formation (%)		Presence of *esp* gene (%)	
	E. faecium	*E. faecalis*	*E. faecium*	*E. faecalis*
Urine (n= 48/25)	38(79.1)	24(96)	36(75)	15(60)
Blood (n=15/06)	12(80)	5(83.3)	14(93.3)	4(66.6)
Pus (n= 22/18)	18(81.8)	16(88.8)	15(68.1)	13(72.2)
High vaginal swab (n=01/0)	1(100)	0(0)	0(0)	0(0)
Tissue (n= 01/02)	1(100)	2(100)	1(100)	1(50)
Catheter Tips (n=03/01)	2(66.7)	1(100)	3(100)	0(0)
Perirectal Swab (n= 02/0)	2(100)	0(0)	2(100)	0(0)

Discussion

Prevalence of enterococcal infections

In our study, the frequency of *E. faecium* was much higher than *E. faecalis*, almost comparable to a report from Pakistan (Yameen *et al.*, 2013). Another report documented *E. faecalis* as most common species among clinical infections from *Enterococcus* species (Sievert *et al.*, 2013).

The same distribution pattern was observed in UK (Molton *et al.*, 2013) and the USA (Hayakawa *et al.*, 2011) with the predominance of *E. faecium* isolates. In our study, most of the enterococci were isolated from urine, pus and blood as reported in some of the previous studies(Bose *et al.*, 2012, Jada and Jayakumar, 2012), suggesting that UTI and bacteremia were the leading infections caused by enterococci.

Prevalence of VRE

The present study revealed a high frequency of VRE (17.48%) in comparison with the previous reports from Pakistan where the frequency was much lower (Abdulla and Abdulla, 2006;Majeed and Izhar, 2006; Yameen *et al*., 2013; Babar *et al*., 2014). This represented a gradual increase in frequency of VRE in Pakistani population which is alarming. This increase in VRE frequency might be due to the excessive and impulsive use of vancomycin. A low frequency of VRE was reported from India (2.1%) (Laishram *et al*., 2014), so as from China (1.5%) (Nateghian *et al*., 2014) and Turkey (3.6%) (Wang *et al*., 2013), as compared to our study. VRE frequency in Iran was more significantly increasing in comparison to other countries of the world (Gozaydin *et al*., 2013, Jones *et al*., 2013, Shokoohizadeh *et al*., 2013). In Europe, VRE frequency ranged from <2% (Finland and Holland) to >20% (Ireland, Portugal, and Greece) (Livermore, 2012, Molton *et al*., 2013). In the UK, the frequency of VRE range was from 17% to 28% whereas in the USA, the prevalence rate was up to 39% (Hayakawa *et al*., 2011).

Resistance profile of VRE

A study on VRE isolates in Canada found 85.8% resistance to ampicillin followed by 2.4% to chloramphenicol, 47.6% to gentamicin and 3% to linezolid (Zhanel *et al*., 2000). In contrast to this report, our VRE isolates showed 100% resistance to ampicillin, gentamicin, rifampicin, ciprofloxacin and norfloxacin followed by teicoplanin (36%), linezolid (44%), chloramphenicol (68%) and nitrofurantoin (76%). These findings were similar to another study from Pakistan with ampicillin (86.36%) and gentamicin (68.18%) whereas 100% susceptibility to linezolid and chloramphenicol (Babar *et al*., 2014). Another study from Pakistan reported VRE with 100% resistance to teicoplanin and none to linezolid (Yameen *et al*., 2013) while from India, low rate of linezolid resistance (3.1%) to VRE was reported (Laishram *et al*., 2014).

All VRE isolates showed high-level vancomycin resistance with 84% VRE isolates with MIC from 128µg/mL to ≥256 µg/mL.

Similar results were cited with 64 µg/mL to 512 µg/mL (Yameen *et al*., 2013) and 64µg/mL to 1024 µg/mL (Shokoohizadeh *et al*., 2013). A Turkish study also reported higher level vancomycin resistance with >256 µg/mL (Gozaydin *et al*., 2013). In this study, the MIC of TEC for 09/25 VRE isolates ranged from 24 µg/mL to 64 µg/mL, although all VRE isolates were found resistant to teicoplanin in another study (Yameen *et al*., 2013). The MIC of linezolid ranged from ≤2µg/mLto 8 µg/mL with approximately similar range for linezolid, documented previously (Laishram *et al*., 2014). In the present study, 2.09% (n=3) enterococci were resistant to TGC via antibiotic disc susceptibility testing but the MIC range was 0.25 µg/mL to 0.42 µg/mL, which is regarded as susceptible according to BSAC (Andrews, 2013). Low MIC value against TGC has already been cited in various reports (Betriu *et al*., 2002, Cercenado *et al*., 2003, Pankey, 2005, Stein andCraig, 2006). Tigecycline resistance is emerging gradually worldwide due to its high uptake (1.8 defined daily dose (DDD) in 2007 to 6.0 DDD in 2010) (Lai *et al*., 2013).

Association of biofilm formation with esp gene

Distribution of biofilm formation and presence of *esp* gene in various specimens was more frequently observed in urine, blood, pus, indwelling medical devices etc but at varying percentages. A number of biofilm forming *E. faecium* and *E. faecalis* were negative for *esp* and vice versa. Kristich *et al*., 2004, confirmed that biofilm formation occurred not only in the absence of *esp*, but in the absence of entire PAIs which harbors *esp* sequence. Mohamed and Murray (2005) found no significant correlation between the presence of *esp* and biofilm formation in a large collection of *E. faecalis* isolates. A number of *esp*-negative *E. faecalis* isolates were found to form moderate to high biofilm (Anderson *et al*., 2016).

Association of biofilm formation with *esp* gene was found insignificant in both VRE and VSE isolates of *E. faecium* and *E. faecalis*, as indicated by χ^2test (*p*-value >0.05). No clear relationship between the expression of *esp* and biofilm formation was found previously (Dworniczek *et al*., 2012).

However, efficient biofilm formation in the absence of *esp* in *E. faecium* isolates has been reported previously (Sillanpää *et al.*, 2010), which confirmed our findings. These findings suggest that there may be some other factors and genes involved in biofilm formation. Biofilm and the *esp* gene are important factors, have a synergy and responsible for the ability of *Enterococci* to colonize and cause nosocomial infections, however, a complete analysis of the association and composition of the biofilm formation of *Enterococciis* proposed.

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