sciendo Revista Română de Medicină de Laborator 2023;31(2):125-134 RESEARCH ARTICLE
DOI:10.2478/rrlm-2023-0013

Vancomycin-resistant enterococci isolated in tertiary care in Southern Thailand: Prevalence and characterization of biofilm formation

Phanvasri Saengsuwan[1*], Kamonnut Singkhamanan[2], Soontara Kawila[3], Chonticha Romyasamit[4]

1. Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Thailand
2. Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine., Prince of Songkla University, Thailand
3. Microbiology Unit, Faculty of Medicine, Prince of Songkla University, Prince of Songkla University, Thailand
4. School of Allied Health Sciences, Walailak University, Thailand

ABSTRACT

Introduction: Vancomycin-resistant enterococci (VRE) are important causative agents of healthcare-associated infections. This study investigated the prevalence of VRE isolates of clinical specimens from a tertiary hospital in Southern Thailand and their biofilm formation and associated virulence factors.

Methods: This retrospective study was conducted from February 2011 to March 2021 at a 1,000-bed tertiary care hospital in Songkhla Province in Southern Thailand.

Results: In total, 95 VRE isolates were collected. Urine had the highest VRE prevalence (38%), followed by pus or secretions (23%) and the digestive tract (19%). VRE infections were most common in medical (45%) and surgical wards (19%). VRE strains were equally resistant (85–100%) to the six classes of antibiotics commonly used in a tertiary care hospital. Biofilms were produced by 73% of the multidrug-resistant strains; however, 93% of VRE isolates lacking hyl were highly capable of producing biofilms.

Conclusions: No relationship was observed between virulence genes and biofilm formation. Thus, efforts to establish appropriate treatment and control measures are necessary, as distinctive VRE characteristics are relevant to the treatment of enterococcal infections in hospitals.

Keywords: antimicrobial resistance, biofilms, confocal laser scanning microscopy, enterococci, virulence

Received: 2 February 2023; Accepted: 9 April 2023; Published: 14 April 2023

INTRODUCTION

Enterococci are facultative anaerobic gram-positive cocci that form pairs or chains and reside in the gastrointestinal tract. Although there are many species of enterococci, the main species causing human diseases are *Enterococcus faecalis* and *Enterococcus faecium*. These can cause various infections, including urinary tract infections, intra-abdominal infections, bacteremia, and endocarditis. Moreover, vancomycin (VAN)-resistant enterococci (VRE) are multidrug-resistant organisms that cause healthcare-associated infections and increase the duration of hospitalization and in-hospital mortalities [1, 2].

Several risk factors are associated with hospital-acquired infections, including ventilator placement, intensive care unit admission, urinary catheterization, surgical incision, prolonged hospitalization, diabetes, and exposure to antimicrobials, particularly carbapenems, fluoroquinolones, ciprofloxacin (CIP), VAN, and piperacillin-tazobactam (PTZ) [3]. High levels of gentamicin (GM) and VAN resistance in *E. faecium* and *E. faecalis* have been identified as a public health concern [4].

Nine pathogenic genes, namely *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*, contribute to the regulation of VAN resistance in enterococci [5]. Collectively, *vanC2* and *vanC3* are known as *vanC2/3*. *VanE* is involved in VAN resistance, but confers sensitivity to teicoplanin. The prevalence of VAN-resistant genes in VRE has been reported in different countries. For example, in Ireland, the prevalence of *vanA* is suspected to be 52% (43/83) [6]. In addition, *vanA* from *Enterococ-*

* Correspondence to: Phanvasri -. Saengsuwan, Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Thailand.
E-mail: sphanvas@medicine.psu.ac.th

cus raffinosus has a prevalence of 58.3% in Bangladesh [7], while *VanB* from *Enterococcus gallinarum* has been identified in India, and its prevalence in China is 3.4% [8, 9]. Genes encoding disease severity factors in enterococci, including those encoding gelatinase (*gelE*), enterococcal surface protein (*esp*), combination factor (*agg*), hyaluronidase (*hyl*), and cytolysin (*cyl*, β-hemolysin), significantly promote enterococcal resistance and persistence and induce biofilm formation. For instance, the Lorestan Hospital in Iran has a high incidence of biofilm-forming *E. faecium* isolates from clinical samples and the environment. Moreover, in India, the biofilm generation rates for *E. faecalis* and *E. faecium* were 27.5 and 17.7%, respectively, in 2019 [10].

An increase in virulence determinants could threaten public health. In a VRE outbreak in Saudi Arabia in 2017, 82.7% of the reported infections were from a university hospital [11]. In 2017, VAN-resistant *E. faecium* was reported by the National Antimicrobial Resistance Surveillance Center of Thailand, Department of Medical Sciences. *E. faecalis*, *E. faecium*, and other *Enterococcus* spp. infection data from patients in 85 hospitals over a 20-year period (2000–2018) showed that these infections accounted for 0.7% and expanded to 9.9% of VRE cases [12]. Consequently, medical treatment costs and mortality rates are high, thereby increasing pressure on the economy. However, data on the characterization of VRE in tertiary hospitals in Southern Thailand remain limited. Therefore, this study aimed to investigate the antibiotic susceptibility patterns, biofilm formation, and virulence factors in VRE recovered from clinical samples to understand the pathogenic potential of these isolates. In addition to assisting in narrowing down the available options for treatment, knowledge of the prevalence and presence of virulence factors, including biofilm production, may aid in comprehending the complicated pathogenesis of VRE. These findings emphasize the importance of implementing strict infection control measures and highlight the role of antimicrobial stewardship of endemic infections in the region.

METHODS

Bacterial isolation

This retrospective study was conducted from February 2011 to March 2021 at a 1,000-bed tertiary care hospital in Songkhla Province in Southern Thailand. Bacterial strains were processed in the Microbiology Unit, Department of Pathology, Faculty of Medicine Prince of Songkla University following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [13]. All VRE isolates were stored in 20% glycerol at −80 °C before use in the experiments. The isolates were identified and confirmed using polymerase chain reaction (PCR) with specific primers for the amplification of *E. faecium ddl*, as described by Dutka-Malen et al. [14]. Data from this study are available in the National Center for Biotechnology Information (NCBI) BioProjects database (PRJNA707345 and PRJNA791465).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion method [13]. The different antibiotic disks (BD GmbH, Heidelberg, Germany) used were as follows: ampicillin (AMP, 10 μg; disk diffusion clinical breakpoints: S ≥ 17; I; R ≤ 16), CIP (5 μg; disk diffusion clinical breakpoints: S ≥ 21; I = 16–20; R ≤ 15), penicillin (PCN, 10 μg; disk diffusion clinical breakpoints: S ≥ 15; R ≤ 14), and VAN (30 μg; disk diffusion clinical breakpoints: S ≥ 17; I = 15-16; R ≤ 14).

Patients admitted with prolonged VRE and mixed infections with other bacteria are difficult to treat and are associated with a high mortality rate and limited therapeutic choices. Therefore, an infectious disease doctor decides on the laboratory determinations of pathogen susceptibility to antibiotics based on minimum inhibitory concentration (MIC) breakpoints for Enterobacterales except for imipenem and meropenem, which are used for *Pseudomonas* infections as defined by CLSI M100 and CLSI 2013 for colistin. In addition, the other antibiotic susceptibilities of the identified gram-negative bacteria were tested against a panel of antibiotics: cefoperazone-sulbactam (SCF, 75/30 μg; disk diffusion clinical breakpoints: S ≥ 21; I = 16–20; R ≤ 15), cefotaxime (30 μg; disk diffusion clinical breakpoints: S ≥ 26; I = 23-25; R ≤ 22), ceftazidime (CAZ, 30 μg; disk diffusion clinical breakpoints: S ≥ 21; I = 18-20; R ≤ 17), ceftriaxone (30 μg; disk diffusion clinical breakpoints: S ≥ 23; I = 20-22; R ≤ 19), colistin (DA, 10 μg; disk diffusion clinical breakpoints: S ≥ 21; I = 15-20; R ≤ 14), ertapenem (ERT, 10 μg; disk diffusion clinical breakpoints: S ≥ 22; I = 19-21; R ≤ 18), GM (120 μg ; disk diffusion clinical breakpoints: S ≥ 10; I = 7-9; R ≤ 6), imipenem (IMP, 10 μg disk diffusion clinical breakpoints: S ≥ 19; I = 16–18; R ≤ 15, according to CLSI M100), meropenem (MEM, 10 μg; disk diffusion clinical breakpoints: S ≥ 19; I = 16–18; R ≤ 15, according to CLSI M100), norfloxacin (NOR, 10 μg; S ≥ 17; I , 13–16; R, ≤ 12), and PTZ (100/10 μg; disk diffusion clinical breakpoints: S ≥ 21; I = 15-20; R ≤ 14). Antimicrobial susceptibility was assessed using inhibition zone diameter. According to these criteria, *Enterococcus* strains were classified as sensitive, intermediate, or resistant.

Multidrug-resistant (MDR) strains were defined as strains resistant to one or more agents in three or more antimicrobial categories; extremely drug-resistant (XDR)

strains were those resistant to at least one agent in all but two or fewer antimicrobial categories; pandrug-resistant strains were those resistant to all agents in all antimicrobial categories [15]. The MICs for AMP- and VAN-resistant strains were ≥16 and ≥8 µg/mL, respectively.

Molecular detection of resistance and virulence genes

DNA extraction was performed according to the manufacturer's instructions using a GF-1 Bacterial DNA Extraction kit (Vivantis Technologies, Selangor Darul Ehsan, Malaysia). Virulence and VAN-resistant genes were detected using specific primers (Table S1) [14-19]. PCR amplification was performed as follows: initial denaturation for 3 min at 94 °C; followed by 35 amplification cycles for 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C; and a final extension for 5 min at 72 °C. A 100-bp DNA ladder (GeneDireX, München, Germany) was used as a molecular-size marker.

E. faecalis ATCC51299 and *E. faecium* ATCC700221 were used as positive controls for *vanB* and *vanA*, respectively. The PCR product was sent for sequencing (1st BASE DNA Sequencing Services, Selangor Darul Ehsan, Malaysia), and sequence similarity was determined using the Basic Local Alignment Search Tool (BLAST) of the NCBI database. All datasets were submitted to the NCBI Sequence Read Archive (BioProject no. PRJNA707345 and PRJNA791465).

Biofilm formation assay

Biofilm formation assays were conducted following previous guidelines [20-21]. Initially, purified *Enterococcus* colonies were resuspended in 10 mL tryptic soy broth (TSB; HI Media, Mumbai, India) and incubated for 18–20 h at 37 °C. Subsequently, for each strain evaluated, 20 µL bacterial suspension was added to three wells of sterile 96-well polystyrene microtiter plates containing 180 µL TSB augmented with 2% glucose. *E. faecium* (ATCC700221) was used as a positive control, and TSB broth (200 µL) was used as the negative control. The microtiter plates were incubated at 37 °C for 24 h, washed with sterile phosphate-buffered solution (PBS), dried at approximately 28–32 °C, and stained with 1% crystal violet for 30 min. The wells were dried after being washed twice with sterile deionized water. Crystal violet dye was resuspended in 150 mL of 99% ethanol after being bound to adherent cells. The optical density (OD) of the solutions in the respective wells was measured at 570 nm using a microtiter plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Each assay was performed in triplicate. Biofilm formation was categorized as negative, weak, moderate, or strong. The mean OD value over three standard deviations (SD) of the negative control was applied to define the cut-off value (ODc): ODc = average OD of the negative control plus (3 SD of the negative control). Each *Enterococcus* isolate was categorized as follows: OD < ODc = non-biofilm producer (category 0); ODc < OD < 2 ODc = weak biofilm producer (category 1); 2 ODc < OD < 4 ODc = moderate biofilm producer (category 2); and OD > 4 ODc = strong biofilm producer (category 3).

Microscopic analysis of biofilms

Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were performed to confirm the biofilms formed by the clinical isolates, and their phenotypic relationship was confirmed using crystal violet staining. SEM was performed as previously described [22], with some modifications. Briefly, biofilms were formed on glass coverslips coated with TSB broth and incubated for 24 h. The coverslips were dried before rinsing twice with PBS and fixed in 2.5% glutaraldehyde for 2 h. Dehydration lasted 30 min and was accomplished using a series of ethanol dilutions (either 25, 50, 75, or 100%). At the end of this process, critical point drying was performed before SEM analysis using a JEOL JSM 5800LV scanning microscope (JEOL, Tokyo, Japan) to acquire biofilm images for qualitative and quantitative analyses through biofilm visualization via CLSM.

CLSM analysis of biofilms

CLSM was performed as previously described [23]. Overnight cultures were adjusted to 5×10^8 CFU/mL with an OD of 0.3 at 600 nm. The biofilms on the glass coverslips were rinsed thrice with PBS after 24 h. The viability of the biofilm cells was evaluated using a LIVE/DEAD BacLight kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To determine the baseline threshold for dead cells, the biofilms were treated with 95% ethanol for 4 h as a negative control. The plates were then incubated in the dark for 15 min at approximately 28–32 °C. After staining, biofilm images were captured using an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan). A 10× water immersion objective (10×/1.2 W) was used to observe biofilms. Three distinct biofilms were used for each condition. Images with 512 × 512 resolution were acquired in at least four different regions of each surface analyzed. Biofilm composition from coverslips to biofilm surfaces was investigated using BioFilmAnalyzer, which is freely available at https://bitbucket.org/rogex/biofilmanalyzer/downloads/ [24].

Ethical statement

This study was approved by the institutional ethics committee of (blinded for review) (REC-63-129-4-8) and was

conducted per the principles of the Helsinki Declaration. Informed consent was waived because the samples were obtained during standard diagnostic care.

Statistical analysis

Demographic data are reported as counts with percentages or median values with the interquartile range (IQR). SPSS Statistics, version 23 (SPSS inc., Chicago, IL, USA), was utilized to analyze all statistical data. The independent samples *t*-test was used to compare the values of continuous variables between groups. Two-tailed *P*-values < 0.05 were considered statistically significant.

RESULTS

Patient characteristics and VRE sources

In total, 95 VRE isolates were obtained from individual patients with enterococcal infection from different samples, including urine (*n* = 36), pus/secretions (*n* = 22; e.g., pancreatic cyst fluid, peritoneal dialysis fluid, and Jackson–Pratt drainage), rectal swabs (*n* = 18), blood (*n* = 10), tissue (*n* = 8), and pelvic (*n* = 1) samples. Of the 95 VRE isolates, 55 (58%) and 40 (42%) were from women and men, respectively, with a female-to-male ratio of 3:2. Moreover, 50% of the VRE-positive patients were aged ≥65 years, with a mean age of 64 years (IQR, 54–63 years; range, 3 months to 90 years). Of these isolates, 43 (45%), 18 (19%), and 11 (12%) were from the medical and surgical wards and intensive care unit, respectively. Most VRE isolates were isolated from urine (*n* = 36, 38%), followed by pus/secretions (*n* = 22, 23%), rectal swabs (*n* = 18, 19%), and blood (*n* = 10, 11%). Additional epidemiological data are presented in Table 1.

Antimicrobial susceptibility patterns

The sensitivity of each VRE isolate to several antimicrobial classes was evaluated. Of the 95 clinical isolates, 72 (76%), 64 (67%), 37 (39%), and <10 (<15%) were tested for susceptibility to AMP; IMP; PCN; and SCF, DA, NOR, CIP, and ERT, respectively. Table 2 presents the antibiotic resistance patterns of the 95 VRE isolates and also lists the antibiotics used in the study. These isolates exhibited high rates of antibiotic resistance; 100, 98, 91, and 96% of the isolates were resistant to VAN, CIP, NOR, and AMP; IMP; cefoperazone; and CAZ, respectively. Of the isolates, 75, 67, and 50% were susceptible to SCF, MEM, and ERT, respectively. High-level resistance to VAN (MIC ≥256 mg/L) was observed in 78 (82%) VRE isolates.

Table 1. Clinical characteristics of patients with VRE infection recruited between February 2011 and March 2021 (n = 95)

Variables	No. of isolates with biofilm phenotype (%)						n	P
	Strong	Medium	Strong or medium	Weak	All positive	Biofilm Negative		
Sex								0.878
Male	4 (10)	4 (10)	8 (20)	22 (55)	30 (75)	10 (25)	40 (42)	
Female	1 (2)	6 (11)	7 (13)	35 (64)	42 (76)	13 (24)	55 (58)	
Age, years								0.097
≤12	0	0	0	1 (25)	1 (25)	3 (75)	4 (4)	
13–24	0	0	0	5 (86)	5 (83)	1 (17)	6 (6)	
25–64	1 (2)	6 (15)	7 (17)	26 (63)	57 (80)	8 (20)	41 (43)	
≥65	4 (9)	4 (9)	8 (18)	25 (57)	33 (75)	11 (25)	44 (46)	
Clinical source								0.573
Urine	1 (3)	4 (11)	5 (14)	20 (56)	25 (69)	11 (31)	36 (38)	
Pus or secretions	2 (9)	0	2 (9)	14 (64)	16 (73)	6 (27)	22 (23)	
Rectal swabs	2 (11)	3 (17)	5 (28)	11 (61)	16 (89)	2 (11)	18 (19)	
Blood	0	0	0	9 (90)	9 (90)	1 (10)	10 (11)	
Tissue	0	3 (38)	3 (25)	2 (25)	5 (63)	3 (38)	8 (8)	
Pelvic	0	0	0	1 (100)	1 (100)	0	1 (1)	
Hospital unit								0.910
Medical wards	4 (9)	5 (12)	9 (21)	23 (53)	32 (74)	11 (26)	43 (45)	
Gynecology ward	0	2 (25)	2 (25)	4 (50)	6 (75)	2 (25)	8 (8)	
Intensive care units	0	1 (9)	1 (9)	7 (64)	8 (73)	3 (27)	11 (12)	
Surgical wards	0	0	0	14 (78)	14 (78)	4 (22)	18 (19)	
Operating room	1 (10)	2 (20)	3 (30)	6 (60)	9 (90)	1 (10)	10 (11)	
Orthopedic ward	0	0	0	1 (50)	1 (50)	1 (50)	2 (2)	
Pediatric ward	0	0	0	2 (67)	2 (67)	1 (33)	3 (3)	
Total	5 (5)	10 (11)	15 (16)	57 (60)	72 (76)	23 (24)	95 (100)	

VRE: Vancomycin-resistant enterococci

Table 2. List of antibiotics used in the study of VRE isolates (*n* = 95)

Antibiotic	n (%)	Susceptibility testing results			P
		Sensitive	Intermediate	Resistant	
Aminoglycoside					..
Gentamicin[1]	20 (21)	7 (35)	0	13 (65)	
Beta-lactamase inhibitors					>0.999
Ampicillin*	72 (76)	0	0	72 (100)	
Penicillin*	37 (39)	0	0	37 (51)	
Piperacillin/tazobactam[2]	12 (13)	0	0	12 (17)	
Carbapenems					0.954
Imipenem[2]	64 (67)	1 (2)	0	63 (98)	
Meropenem[2]	15 (16)	10 (67)	0	5 (33)	
Ertapenem[2]	2 (2)	1 (50)	0	1 (50)	
Cephalosporin					<0.001
Cefotaxime[1]	13 (14)	2 (15)	0	11 (85)	
Ceftazidime[1]	14 (15)	2 (14)	0	12 (86)	
Ceftriaxone[1]	12 (13)	2 (17)	0	10 (83)	
Cefoperazone[1]	11 (12)	1 (9)	0	10 (91)	
Cefoperazone-sulbactam[1]	8 (8)	6 (75)	2 (25)	0	
Fluoroquinolone					>0.999
Ciprofloxacin*	2 (2)	0	0	2 (100)	
Norfloxacin[2]	5 (5)	0	0	5 (100)	
Glycopeptide					..
Vancomycin*	95 (100)	0	0	95 (100)	
Polymyxin					..
Colistin[2]	8 (8)	4 (50)	0	4 (50)	

VRE: Vancomycin-resistant enterococci; * CLSI antibiotics; [1]High-level aminoglycoside resistance is interpreted as a positive or negative result based on the indicated cutoff values. The CLSI recommends reading gentamicin disk diffusion results at 16 to 18h, gentamicin broth or agar dilution methods results at 24h (reincubation required if susceptible at 24h); [2] non-CLSI antibiotics were tested due to the inhibition zone diameter. Please see CLSI M100 document guidelines for further detailed recommendations on testing for each agent.

Detection of resistance and virulence factor genes in VRE

VanA was detected in all 95 VRE strains; however, *vanB*, *vanC*, *vanD*, *vanE*, and *vanF* were not detected. *Esp* and *hyl* were identified in 87 (92%) and 7 (7%) of the isolates, respectively. However, none of the isolates harbored *gel*, *cyt*, *cpd*, or *ebp*. Table S1 shows the amplicons of the VRE isolates and the distribution of the resistance genes.

Genes associated with biofilm formation and their effects

The 95 VRE isolates were classified based on the extent of biofilm formation as follows: non-formers (*n* = 23, 24%), weak formers (*n* = 57, 60%), moderate formers (*n* = 10, 11%), and strong formers (*n* = 5, 7%). Biofilm formers accounted for 76% (*n* = 72) of all positive isolates, 73% (*n* = 69) of MDR-VRE isolates, and 27% (*n* = 16) of XDR-VRE isolates. Regarding the effect of virulence factor genes, *esp*+ isolates produced more biofilms than those for *hyl*+ isolates, whereas *hyl*− isolates produced more biofilms than those of *esp*− isolates; however, no significant association was observed between virulence factor genes and biofilm formation (Table 3).

Additionally, clinical strains formed more biofilms than the ATCC700221 strain (Figure 1). Notably, clinical strains harboring *esp* genes and a strong biofilm phenotype had significantly higher biomass than those of the ATCC700221 strain and the strain with a weak biofilm phenotype (Figure 2).

DISCUSSION

This study revealed that the distribution of VRE isolates differed according to the infection site. Most clinical isolates were recovered from urine at 38%, which is lower than the 69.1% VRE prevalence in urine recorded in Taiwan [25]. In the present study, the highest number of isolates was from patients hospitalized in a medical ward (45%). Differences in the frequency of VRE infections have also been reported. For example, Karki et al. [26] reported that VRE infections ranged from 8 to 29% in inpatient wards in Australia. The regional disparities revealed in this study highlight that infection control for resistant pathogens should be based on local epidemiology.

We found that 73% of VRE strains were MDR, with the remaining 27% being XDR isolates. The prevalence of MDR-VRE was 1.5-fold higher in female inpatients than that in male inpatients, which contrasts with the results of a review of nine studies reporting that 59% of the patients with VRE bloodstream infections were men, with a male-to-female prevalence ratio of 1:4 [27]. These discrepancies might be attributed to the differences in the study design and population.

Table 3. Relationship between biofilm-forming capacity and drug susceptibility patterns of VRE infection, resistance genes, and virulence factors

	No. of isolates with biofilm phenotype (%)							
Type	**Strong**	**Medium**	**Strong or medium**	**Weak**	**All positive**	**Negative**	**Total n**	**P**
Pattern								0.218
XDR-VRE	0	4 (15)	4 (15)	18 (69)	22 (85)	4 (15)	26 (27)	
MDR-VRE	5 (7)	6 (9)	11 (16)	39 (57)	50 (72)	19 (28)	69 (73)	
Total	5 (7)	10 (11)	15 (16)	57 (60)	72 (76)	23 (24)	95 (100)	
Resistance genes								
vanA	5 (5)	10 (11)	15 (16)	57 (60)	72 (76)	23 (24)	95 (100)	-
Virulence factors								
esp+	5 (6)	9 (11)	14 (17)	50 (57)	64 (74)	23 (26)	87 (92)	0.538
esp−	0	1 (13)	1(13)	7 (88)	8 (100)	0	8 (8)	
hyl+	1 (14)	1 (14)	2 (29)	4 (57)	6 (86)	1 (14)	7 (7)	0.431
hyl−	4 (5)	9 (10)	13 (15)	53 (60)	66 (75)	22 (25)	88 (93)	

VRE: Vancomycin-resistant enterococci

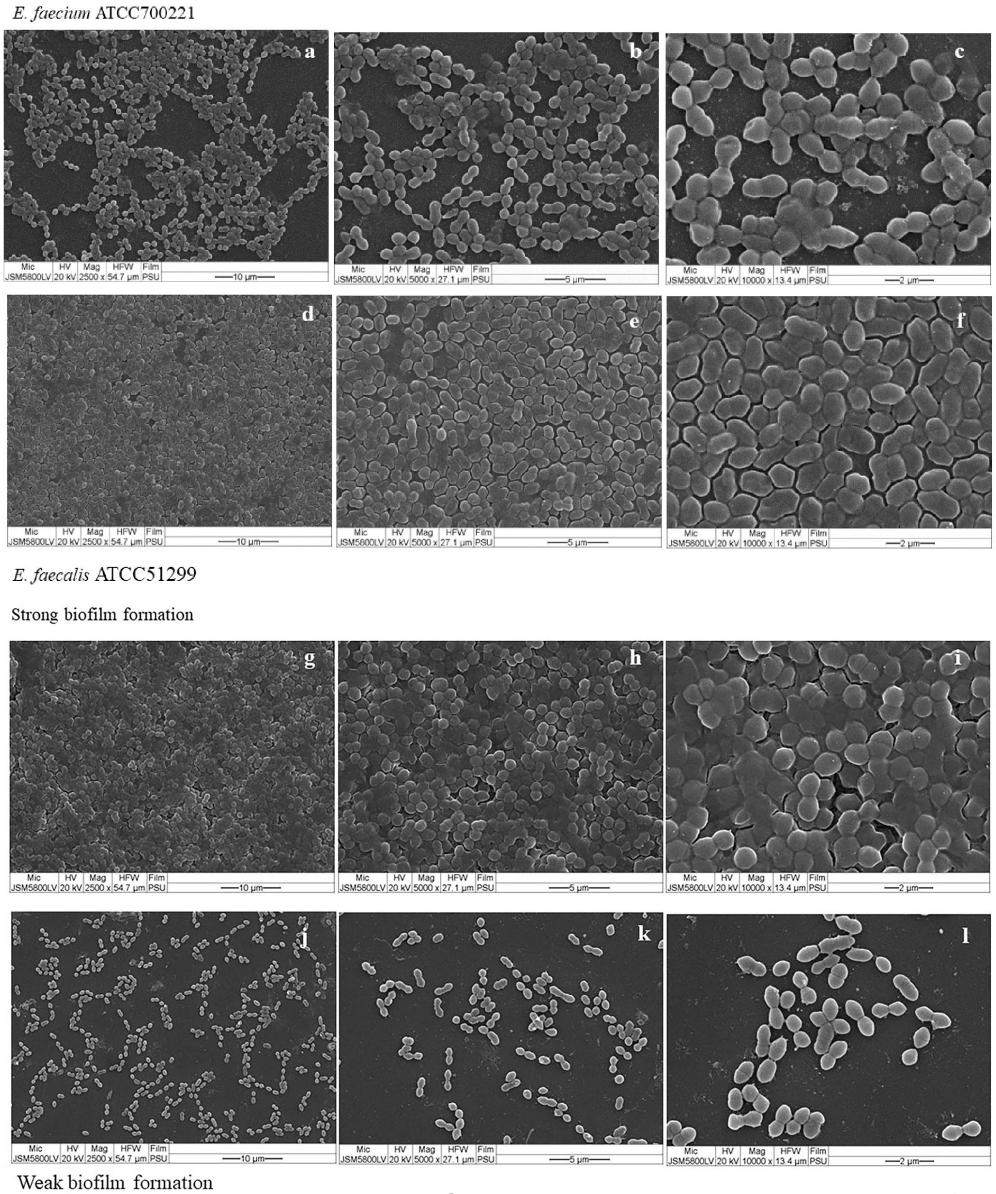
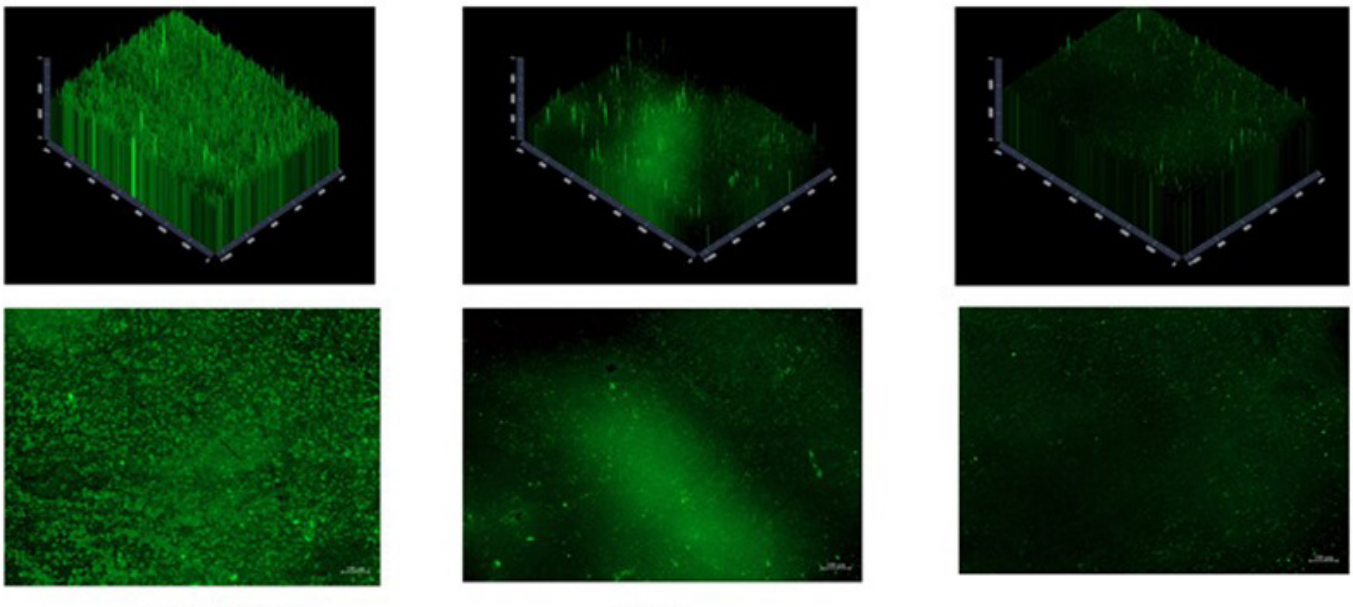


Fig. 1. Scanning electron microscopy images of vancomycin-resistant enterococci isolates. Enterococcus faecium (a–c) ATCC700221 and Enterococcus faecalis (d–f) ATCC51299. Clinical strain with strong biofilm formation (g–i); clinical strain with weak biofilm formation (j–l). Magnification 2,500×, 5,000× , and 10,000×; Bars = 10 µm.



	No. of effective cells (μm^{-3}), mean ± SD	*P*
E. faecium ATCC700221	44.33 ± 10.69	0.0003
1VRE	1,734.33 ± 47.23	0.0497
2VRE	1,038.67 ± 320.86	0.0319

Fig. 2. Confocal laser scanning microscopy images of Enterococcus faecium ATCC700221 and clinical strain biofilms (1VRE and 2VRE). Bio-volume of bacteria biofilms were quantified by Biofilm Analyzer software (https://bitbucket.org/rogex/biofilmanalyzer/downloads/). The results shown are the mean and standard deviation of three independent experiments. VRE: Vancomycin-resistant enterococci

All isolates were resistant to VAN (*n* = 95) and AMP (*n* = 72). These VRE isolates exhibited 98, 50, and 33% susceptibility to IMP, ERT, and MEM, respectively, corresponding with previously reported findings [4]. Notably, the synergistic combination of SCF (cephalosporin drugs) and carbapenems may aid in treating enterococcal infections [28]. The extensive use of antibiotics in hospitals could result in the rise of antibiotic-resistant enterococci in this region. Further studies are warranted to determine the rates of resistance to different antibiotics among enterococci and mixed infections with other bacteria, and to assess their clinical efficacy. While selecting an antibiotic, it is also important to remember that various nations have distinct antibiotic standards. Furthermore, regional recommendations and legal restrictions should be considered. Importantly, antibiotics considered critical should be used with extreme caution and never as first-line drugs.

In the present study, *vanA* was present in all VRE isolates (95, 100%), whereas *vanB*, *vanC*, *vanD*, *vanE*, and *vanF* were not detected, which is consistent with previous reports, where the prevalence of *vanA* in VRE isolates was 90% in the northwest of Iran [29] and 84% in Trinidad and Tobago [30]. *VanA* may be the most clinically important genotype and can confer high levels of resistance to VAN and teicoplanin [31].

Although *E. faecalis* is normally less drug-resistant than *E. faecium*, we discovered that biofilm formation was more common in VRE isolates than previously described for *E. faecalis* [32]. The prevalence of VRE biofilms in this study was 76%, which is consistent with the 80.36% prevalence in *Enterococcus* isolated from humans in Poland [33] and greater than the 28% reported earlier in northern India [34]. In our study, MDR-VRE isolates were 2.5-fold more likely to produce biofilms than XDR-VRE isolates. These clinical isolates carry virulence factors involved in biofilm formation, and chronic infection with these strains typically causes severe disease with limited treatment options [35]. The increased prevalence of VRE, particularly VAN-resistant *E. faecium*, in various countries has been linked to the emergence and spread of *vanA*- and *vanB*-positive VRE with virulence factors, such as enterococcal surface protein (ESP) (*esp*), cytolysin (*cyl*), and hyaluronidase (*hyl*). ESP is a cell wall-associated protein encoded by *esp* and was discovered on a pathogenicity island in MDR pathogenic *E. faecalis*

and *E. faecium* strains [36]. Furthermore, ESP aids in the colonization and persistence of *E. faecalis* strains in increasing urinary tract infections and/or endocarditis and may contribute to biofilm formation and antimicrobial resistance. In the current study, the prevalence of VRE carrying *esp* was 74% (*n* = 64), which is consistent with findings from Brazil, where 70% of 240 enterococci samples carried *esp*, with *E. faecalis* and *E. faecium* accounting for 70.1 and 68.4%, respectively [37]. The expression level of *esp* varies depending on growth conditions, VRE strain, and country and is associated with initial connection and biofilm formation [38].

Notably, some VRE isolates lacking certain virulence genes formed biofilms (75–100%). Furthermore, the biomass from the clinical specimens was higher than that from the ATCC strain and clinical isolates with weak-biofilm-former phenotypes.

We found a higher-than-usual frequency (approximately 96%) of biofilm formation, regardless of its strength, in VRE clinical isolates recovered from a tertiary hospital in Southern Thailand. This frequency is considerably higher than those reported in other developing countries, where 68% of isolates in Tamil Nadu, India [39], and 64.40% of isolates from urinary tract infections in a hospital in Dhaka, Bangladesh, were biofilm producers [40]. Moreover, we found that the ability to form biofilms did not correlate with the presence of *esp*, which is consistent with previous findings, where biofilm formation in *Enterococcus* isolates was not correlated with the presence of *gel* and *esp* [11]. Therefore, ESP is not crucial for biofilm formation, and more biofilm-associated proteins, such as those encoded by *gelE* and *agg*, might be involved.

This study has some limitations. First, it was conducted in a single institution, and the sample size of VRE isolates was relatively small. Second, we could not collect patient information, including chronic conditions and other clinical data. Third, we did not investigate the antimicrobial resistance mechanisms or their potential interactions with virulence factor genes, which we intend to explore in future studies. Further studies with large sample sizes are required to investigate the relationship between biofilm production and other risk factors.

CONCLUSIONS

Biofilm production is an important virulence factor among MDR enterococcal isolates, and their eradication is extremely difficult. VAN, beta-lactamase inhibitors, and fluoroquinolones may be ineffective against VRE infections in our region. In contrast, MEM and ERT are effective against VRE strains, and SCF can be used as an alternative to treat enterococcal infections. The molecular approach and SEM/CLSM are useful tools for detecting resistance and virulence genes and biofilm formation and should be used more regularly. With the considerable interest in Enterococcus as a potent pathogen, identifying factors associated with invasiveness and systemic diseases has become essential, and further investigation is required. Although we did not find a relationship between the presence of virulence genes and biofilm formation, awareness about VRE infections and vigilance in hospitals is necessary to control and prevent their spread to the environment.

ABBREVIATIONS

AMP – ampicillin
CAZ – ceftazidime
CIP – ciprofloxacin
CLSM – confocal laser scanning microscopy
DA – colistin
ERT – ertapenem
GM – gentamicin
I – intermediate
IMP – imipenem
IQR – interquartile range
MDR – multidrug-resistant
MEM – meropenem
MIC – minimal inhibitory concentration
NCBI – National Center for Biotechnology Information
NOR – norfloxacin
OD – optical density
PBS – phosphate-buffered solution
PCN – penicillin
PCR – polymerase chain reaction
PTZ – piperacillin-tazobactam
R – resistant
S – susceptible
SCF – cefoperazone-sulbactam
SD – standard deviation
SEM – scanning electron microscopy
TSB – tryptic soy broth
VAN – vancomycin
VRE – vancomycin-resistant enterococci
XDR – extremely drug-resistant

ACKNOWLEDGMENT

This work was supported by the Faculty of Medicine, Prince of Songkla University, grant number 63-129-4-8.

The authors gratefully acknowledge Editage (https://www.editage.com/) for English language editing.

AUTHORS' CONTRIBUTION

PS – Conceptualization, funding acquisition, data curation, writing – original draft preparation, writing – reviewing and editing

KS –Data curation

SK – Investigation

CR – Investigation, data curation

The manuscript, as submitted, has been reviewed by and approved by all the authors.

CONFLICT OF INTEREST

None to declare.

PUBLISHER'S NOTE

Editorial Office stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. O'Driscoll T, Crank CW. Vancomycin-resistant enterococcal infections:Epidemiology, clinical manifestations, and optimal management. Infect Drug Resist. 2015;8:217-30. DOI: 10.2147/IDR.S54125
2. Chen YP, Tasi XW, Chang K, Cao XD, Chen JR, Liao CS. Multi-drug resistant organisms infection impact on patients length of stay in respiratory care ward. Antibiotics (Basel). 2021;10(5):608. DOI: 10.3390/antibiotics10050608
3. Donlan RM. Biofilm formation:a clinically relevant microbiological process. Clin Infect Dis. 2001;33(8):1387-92. DOI: 10.1086/322972
4. Sparo M, Delpech G, García Allende N. Impact on public health of the spread of high-level resistance to gentamicin and vancomycin in enterococci. Front Microbiol. 2018;9:3073. DOI: 10.3389/fmicb.2018.03073
5. Protonotariou E, Dimitroulia E, Pournaras S, Pitiriga V, Sofianou D, Tsakris A. Trends in antimicrobial resistance of clinical isolates of Enterococcus faecalis and Enterococcus faecium in Greece between 2002 and 2007. J Hosp Infect. 2010;75(3):225-7. DOI: 10.1016/j.jhin.2009.12.007
6. Jolivet S, Fines-Guyon M, Nebbad B, Merle JC, Le Pluart D, Brun-Buisson C, et al. First nosocomial outbreak of vanA-type vancomycin-resistant Enterococcus raffinosus in France. J Hosp Infect. 2016;94(4):346-50. DOI: 10.1016/j.jhin.2016.09.004
7. Islam TAB, Shamsuzzaman SM. Isolation and species identification of enterococci from clinical specimen with their antimicrobial susceptibility pattern in a tertiary care hospital, Bangladesh. J Coast Life Med. 2015;3(10):787-90. DOI: 10.12980/jclm.3.2015j5-95
8. Biswas PP, Dey S, Adhikari L, Sen A. Detection of vancomycin resistance in Enterococcus species isolated from clinical samples and feces of colonized patients by phenotypic and genotypic methods. Indian J Pathol Microbiol. 2016;59(2):188-93. DOI: 10.4103/0377-4929.182015
9. Zhao C, Sun H, Wang H, Liu Y, Hu B, Yu Y, et al. Antimicrobial resistance trends among 5608 clinical Gram-positive isolates in China:results from the Gram-positive Cocci Resistance Surveillance program (2005-2010). Diagn Microbiol Infect Dis. 2012;73(2):174-81. DOI: 10.1016/j.diagmicrobio.2012.03.003
10. Shridhar S, Dhanashree B. Antibiotic susceptibility pattern and biofilm formation in clinical isolates of Enterococcus spp. Interdiscip Perspect Infect Dis. 2019;2019:7854968. DOI: 10.1155/2019/7854968
11. Alotaibi FE, Bukhari EE. Emergence of vancomycin-resistant enterococci at a teaching hospital, Saudi Arabia. Chin Med J (Engl). 2017;130(3):340-6. DOI: 10.4103/0366-6999.198923
12. National Antimicrobial Resistance Surveillance Thailand. Antimicrobial resistance rate of Enterococcus spp. by year (NARST-92 hospitals, 12M 2019), http://narst.dmsc.moph.go.th/data/AMR%202000-2019-12M.pdf [accessed 16 September 2020].
13. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 29th ed. CLSI supplement M100. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute;2019.
14. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995;33(5):24-7. DOI: 10.1128/jcm.33.1.24-27.1995
15. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria:an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268-81. DOI: 10.1111/j.1469-0691.2011.03570.x
16. Elsner HA, Sobottka I, Mack D, Claussen M, Laufs R, Wirth R. Virulence factors of Enterococcus faecalis and Enterococcus faecium blood culture isolates. Eur J Clin Microbiol Infect Dis. 2000;19(1):39-42. DOI: 10.1007/s100960050007
17. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, et al. Development of a multiplex PCR for the detection of asa1, gelE, cylA, esp, and hyl genes in enterococci and survey for virulence determinants among European hospital isolates of Enterococcus faecium. J Clin Microbiol. 2004;42(10):4473-9. DOI: 10.1128/JCM.42.10.4473-4479.2004
18. Bourgogne A, Singh KV, Fox KA, Pflughoeft KJ, Murray BE, Garsin DA. EbpR is important for biofilm formation by activating expression of the endocarditis and biofilm-associated pilus operon (ebpABC) of Enterococcus faecalis OG1RF. J Bacteriol. 2007;189(17):6490-3. DOI: 10.1128/JB.00594-07
19. Bhatt P, Sahni AK, Praharaj AK, Grover N, Kumar M, Chaudhari CN, et al. Detection of glycopeptide resistance genes in enterococci by multiplex PCR. Med J Armed Forces India. 2015;71(1):43-7. DOI: 10.1016/j.mjafi.2014.03.005
20. Kafil HS, Mobarez AM. Assessment of biofilm formation by enterococci isolates from urinary tract infections with different virulence profiles. J King Saud Univ Sci. 2015;27(4):312-7. DOI: 10.1016/j.jksus.2014.12.007
21. Cui P, Feng L, Zhang L, He J, An T, Fu X, et al. Antimicrobial Resistance, Virulence Genes, and Biofilm Formation Capacity Among Enterococcus species From Yaks in Aba Tibetan Autonomous Prefecture, China. Front Microbiol. 2020;11. DOI: 10.3389/fmicb.2020.01250

22. Vickery K, Deva A, Jacombs A, Allan J, Valente P, Gosbell IB. Presence of biofilm containing viable multiresistant organisms despite terminal cleaning on clinical surfaces in an intensive care unit. J Hosp Infect. 2012;80(1):52-5. DOI: 10.1016/j.jhin.2011.07.007
23. Puchter L, Chaberny IF, Schwab F, Vonberg RP, Bange FC, Ebadi E. Economic burden of nosocomial infections caused by vancomycin-resistant enterococci. Antimicrob Resist Infect Control. 2018;7:1. DOI: 10.1186/s13756-017-0291-z
24. Bogachev MI, Volkov VY, Markelov OA, Trizna EY, Baydamshina DR, Melnikov V, et al. Fast and simple tool for the quantification of biofilm-embedded cells sub-populations from fluorescent microscopic images. PLoS One. 2018;13(5):e0193267. DOI: 10.1371/journal.pone.0193267
25. Lee MC, Lu CH, Lee WY, Lee CM. Correlation between nosocomial carriage of vancomycin-resistant enterococci and antimicrobial use in Taiwan. Am J Trop Med Hyg. 2020;104(3):1131-6. DOI: 10.4269/ajtmh.20-0842
26. Karki S, Houston L, Land G, Bass P, Kehoe R, Borrell S, et al. Prevalence and risk factors for VRE colonisation in a tertiary hospital in Melbourne, Australia:a cross sectional study. Antimicrob Resist Infect Control. 2012;1(1):31. DOI: 10.1186/2047-2994-1-31
27. Shahi F, Hamidi H, Khoshnood S, Mehdipour G, Dezfouli A, Sheikh A. Virulence determinants and biofilm formation in clinical isolates of Enterococcus:a cross-sectional study. J Acute Dis. 2020;9(1):27-32. DOI: 10.4103/2221-6189.276079
28. Gagetti P, Bonofiglio L, García Gabarrot G, Kaufman S, Mollerach M, Vigliarolo L, et al. Resistance to β-lactams in enterococci. Rev Argent Microbiol. 2019;51(2):179-83. DOI: 10.1016/j.ram.2018.01.007
29. Jahansepas A, Ahangarzadeh Rezaee M, Hasani A, Sharifi Y, Rahnamaye Farzami M, Dolatyar A, et al. Molecular epidemiology of vancomycin-resistant Enterococcus faecalis and Enterococcus faecium isolated from clinical specimens in the northwest of Iran. Microb Drug Resist. 2018;24(8):1165-73. DOI: 10.1089/mdr.2017.0380
30. Akpaka PE, Kissoon S, Jayaratne P. Molecular analysis of vancomycin-resistant enterococci isolated from regional hospitals in Trinidad and Tobago. Adv Med. 2016;2016:8762691. DOI: 10.1155/2016/8762691
31. Ahmed MO, Baptiste KE. Vancomycin-resistant enterococci:a review of antimicrobial resistance mechanisms and perspectives of human and animal health. Microb Drug Resist. 2018;24(5):590-606. DOI: 10.1089/mdr.2017.0147
32. Zheng JX, Bai B, Lin ZW, Pu ZY, Yao WM, Chen Z, et al. Characterization of biofilm formation by Enterococcus faecalis isolates derived from urinary tract infections in China. J Med Microbiol. 2018;67(1):60-7. DOI: 10.1099/jmm.0.000647
33. Woźniak-Biel A, Bugla-Płoskońska G, Burdzy J, Korzekwa K, Ploch S, Wieliczko A. Antimicrobial resistance and biofilm formation in Enterococcus spp. Isolated from humans and turkeys in Poland. Microb Drug Resist. 2019;25(2):277-86. DOI: 10.1089/mdr.2018.0221
34. Das AK, Dudeja M, Kohli S, Ray P, Singh M, Kaur PS. Biofilm synthesis and other virulence factors in multidrug- resistant uropathogenic enterococci isolated in Northern India. Indian J Med Microbiol. 2020;38(2):200-9. DOI: 10.4103/ijmm.IJMM_19_355
35. Saffari F, Dalfardi MS, Mansouri S, Ahmadrajabi R. Survey for correlation between biofilm formation and virulence determinants in a collection of pathogenic and fecal Enterococcus faecalis isolates. Infect Chemother. 2017;49(3):176-83. DOI: 10.3947/ic.2017.49.3.176
36. Comerlato CB, Resende MC, Caierão J, d'Azevedo PA. Presence of virulence factors in Enterococcus faecalis and Enterococcus faecium susceptible and resistant to vancomycin. Mem Inst Oswaldo Cruz. 2013;108(5):590-5. DOI: 10.1590/S0074-02762013000500009
37. Soares RO, Fedi AC, Reiter KC, Caierão J, d'Azevedo PA. Correlation between biofilm formation and gelE, esp, and agg genes in Enterococcus spp. clinical isolates. Virulence. 2014;5(5):634-7.d DOI: 10.4161/viru.28998
38. Van Wamel WJ, Hendrickx AP, Bonten MJ, Top J, Posthuma G, Willems RJ. Growth condition-dependent Esp expression by Enterococcus faecium affects initial adherence and biofilm formation. Infect Immun. 2007;75(2):924-31. DOI: 10.1128/IAI.00941-06
39. Sindhanai V, Avanthiga S, Suresh Chander V. Antibiotic susceptibility pattern of biofilm forming and biofilm non forming enterococci species. IOSR JDMS. 2016;15(4):33-7.
40. Akhter J, Ahmed S, Saleh AA, Anwar S. Antimicrobial resistance and in vitro biofilm-forming ability of Enterococci spp. isolated from urinary tract infection in a tertiary care hospital in Dhaka. Bangladesh Med Res Counc Bull. 2014;40(1):6-9. DOI: 10.3329/bmrcb.v40i1.20320