ORIGINAL ARTICLE

Linezolid Susceptibility and Virulence Factors in Vancomycin Resistant *Enterococcus faecalis* and *Enterococcus faecium* among Hospitalized Burn Patients

[1]Shymaa A. El-Askary*, [2]Eman M. Zaher
[1]Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University
[2]Clinical pathology, Faculty of Medicine, Menoufia University

Key words:
Enterococcus, VRE, LZD, and Virulence

****Corresponding Author:***
Shymaa Abd Elsattar Elaskary
Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University
Tel.: 01025538299
dr.shaimaaelaskary@yahoo.com

ABSTRACT

Background: *Enterococci are the 3rd cause of HAIs. E. faecalis and E. faecium are the commonest enterococcal species, showed resistance to vancomycin due to resistance genes (vanA, vanB and vanC). Linezolid is considered a* good substitute. *The virulence factors like asa1, gelE, cylA, esp, and hyl may interfere with antibiotic susceptibility.* ***Objectives:*** *Determine linezolid resistance in VR E. faecalis and E. faecium in relation to virulence factors.* ***Methodology:*** *Enterococcus* spp. *identified by colony morphology, Gram stain, biochemical reactions and by the VITEK 2 system. Antibiotic susceptibility was done through VITEK 2 system, AST-GP72 card. Vancomycin and linezolid MIC were done according to CLSI. Multiplex PCR for* $ddl_{E.\ faecalis}$, $ddl_{E.\ faecium}$. *vanA and vanB detection. Other for asa1, gelE, cylA, esp, and hyl virulence genes determination then conventional PCR for cfr and optrA genes were done.* ***Results:*** *A total of 65 enterococci CIs. (45 E. faecalis & 20 E. faecium) were isolated from different samples. E. faecalis and E. faecium were resistant to vancomycin by 11,1% and 35% and to linezolid by 4.4% and 10% respectively. The vanA, vanB, cfr and optrA genes were present in 100% of VR E. faecalis like E. faecium except that, the cfr was not detected. The gelE was frequently detected in E. faecalis followed by asa1, esp, hyl and finally cylA. And for E. faecium, the most frequent one was asa1followed by gelE. esp, and finally cylA and hyl.* ***Conclusions:*** *LZD resistant enterococci were increasingly detected, with no significant relation between linezolid resistance and vancomycin resistance. And with different impact of virulence genes.*

INTRODUCTION

Currently enterococci, Gram-positive bacteria that are component of the usual fora of the human gut, were considered as the third recorded cause of nosocomial infections[1]. Since enterococci can acquire antibiotic resistance genes, they become naturally resistant to many antibiotics and can cause infection in wounds, including burn wounds[2].

E. faecalis and *E. faecium* are the commonest enterococcal species, reporting about 90% of enterococcal infections, followed by other enterococcal species[3].

Initially *E. faecalis* was almost accountable for 90% of human enterococcal infections in hospitals, and only 10% were caused by *E. faecium*[4,5]**.** However, recently, *E. faecium*, becomes much more frequently incorporated in nosocomial infections due to their resistance to ampicillin, vancomycin, and high levels of aminoglycosides than *E. faecalis*[6].

Resistance of enterococci to glycopeptide is now considered a major clinical interest. Three glycopeptide resistance phenotypes occurred based on the degree of vancomycin and teicoplanin resistance[7]. The *vanA* type accounts for vancomycin and teicoplanin resistance[8]. The *vanB* type is responsible for resistance to different levels of vancomycin but not to teicoplanin[9], and the *vanC* type for low-level resistance to vancomycin[10].

Since, enterococci with vancomycin resistance (VRE) have been described as a primary source of nosocomial outbreaks[11]. Linezolid (LZD), the first synthetic antimicrobial agent of oxazolidinone class, prevents the early ribosome assembly and protein synthesis of several gram-positive bacterial species, directing the 50S ribosome subunits and affecting its binding with formyl methionyl-tRNA is considered good substitute for those infections control[12] , however, enterococci resistant to linezolid have been developed and mediated by several resistance genes, including *ermA*, *ermB*, *ermC*, *tetM*, , *cfr*, *cfrB*, *poxtA*, as well as *optrA*[3, 13, 14].

Enterococci have numerous virulence factors that may interfere with their antibiotic susceptibility, for *E. faecalis*, there are aggregation substance encoded by plasmid *asa1*[15], gelatinase encoded by the chromosomal *gelE*[16] and cytolysin encoded by plasmid or chromosomally integrated genes[17]. For *E. faecium*, the

enterococcal surface protein that encoded by the chromosomal *esp*,[18], and, very newly, hyaluronidase encoded by the chromosomal *hyl*[19,20,21,22].

The aim of this study is to determine the frequency of linezolid resistance in VR *E. faecalis* and *E. faecium* isolated from hospitalized burn patients in relation to different enterococcal virulence factors.

METHODOLOGY

Samples collection:

A total of 65 enterococci CIs. (45 *E. faecalis* & 20 *E. faecium*) were collected from different clinical samples of burn patients admitted to Menoufia University Hospitals (MUH) in the Medical Microbiology and Immunology Department Laboratory during the period from January 2021 to December 2021.The study design was approved by the ethical committee, Faculty of Medicine, Menoufia University.

Isolation and Identification:

E. faecalis and *E. faecium* clinical isolates (CIs.) were isolated from wound, blood and urine samples and identified by colony morphology, Gram stain, biochemical reactions and by the VITEK 2 system (*BioMérieux, Marcyl'Etoile, France)*[14]. Additionally, species confirmation was done by PCR using specifc primers (*ddl* $_{E.\ faecalis}$ and *ddl* $_{E.\ faecium}$)[2].

Antibiotic susceptibility testing and detection of vancomycin and linezolid resistance.

The antimicrobial susceptibility of numerous frequently used antibiotics, such as Ampicillin, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin (high level), Linezolid, Nitrofurantoin, Quinupristin-dalfopristin, Streptomycin (high level), Tigecycline and Vancomycin were automatically tested through VITEK 2 compact system, AST-GP72 card (*BioMérieux, France*). *E. faecalis* ATCC 29212 was the quality control (QC) strains tested with each run. The susceptibility breakpoints of these antibiotics in *E. faecalis* and *E. faecium* were optimised according to Clinical & Laboratory Standards Institute (CLSI)[23] (**Fig.1**).

The minimum inhibitory concentration (MIC) for vancomycin & linezolid was determined by standard broth microdilution method, as per CLSI guidelines[24]. *E. faecalis* ATCC 29212 was used as control strain. Where the vancomycin susceptible breakpoint was assumed as ≤ 4µg/mL for susceptibility, 8-16µg/mL for intermediate status, and ≥32µg/mL for resistance. And for linezolid, the susceptible breakpoint was: ≤2µg/mL for susceptibility, 4µg/mL for intermediate status, and ≥8µg/mL for resistance[24].

Multiplex PCR analysis of vancomycin-resistant *E. faecium* and *E. faecalis* species:

The whole genomic DNA was extracted as mentioned before[20]. Concurrent detection of genes encoding D-alanine–D-alanine ligases specific for *E. faecalis* (*ddl*$_{E.\ faecalis}$) and *E. faecium* (*ddl*$_{E.\ faecium}$) and glycopeptide-resistance genotypes (*vanA*, *vanB*) [25] is shown in table1& fig.2a.

Multiplex PCR analysis for detection of *E. faecium* and *E. faecalis* virulence genes. *asa1*, *gelE*, *cylA*, *esp*, and *hyl* as previously described [20] is presented in table1& fig.2b.

Conventional PCR for detection of *cfr* and *optrA* linezolid resistance genes. as previously described [26,27] is shown in table1 & fig.2c.

Table 1: Genes used in the study with their nucleotide sequence and size.

Gene name	Oligonucleotide sequence (5' to 3')	Product size (bp)	Reference No.
ddl $_{E.\ faecalis}$	ATCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	941	**25**
ddl $_{E.\ faecium}$	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	658	
Van A	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1,030	
Van B	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433	
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	375	**20**
gelE	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213	
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688	
esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG	510	
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA	276	
cfr	TGTATGTTTTGACTTTC ATTATCTTCCACCCAGTAGTC	1,320	**3**
optrA	AGGTGGTCAGCGAACTA ATCAACTGTTCCCATTC	1,395	

Statistical analysis of the collected data:

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. **(**Armonk, NY: IBM Corp**)**. Categorical data were represented as numbers and percentages. **Chi-square test** was applied to investigate the association between the categorical variables. **Z test** was used to compare two proportions (percentages) in the same or different groups. Significance of the obtained results was judged at the 5% level

RESULTS

In our study, out of 270 burn patients admitted to Menoufia University hospital, *Enterococcus* spp. was isolated from 24.1% (n=65/270) of the burn patient clinical isolates among them *E. faecalis* was 45 (69.2%) and *E. faecium* was 20 (30.8%) distributed in wound, blood, and urine specimens as 40% (n=18), 37.7% (n=17) and 22.3% (n=10) for *E. faecalis* and 50% (n=10), 20% (n=4) and 30% (n=6) for *E. faecium* respectively.

Resistance to Ampicillin, Ciprofloxacin, Daptomycin, Erythromycin, Gentamicin (high level), Linezolid, Nitrofurantoin, Quinupristin-dalfopristin, Streptomycin (high level), Tigecycline and Vancomycin was 11.8%, 64.6%, 75.5%, 93.3%, 55.6%, 4.4%, 86.6%, 75.5%, 73.3%, 73.3%, 11.1% for *E. faecalis* and 25%, 55%, 15%, 95%, 85%, 10%, 90%, 85%, 65%, 15%, 35% for *E. faecium* respectively (**Fig.1**).

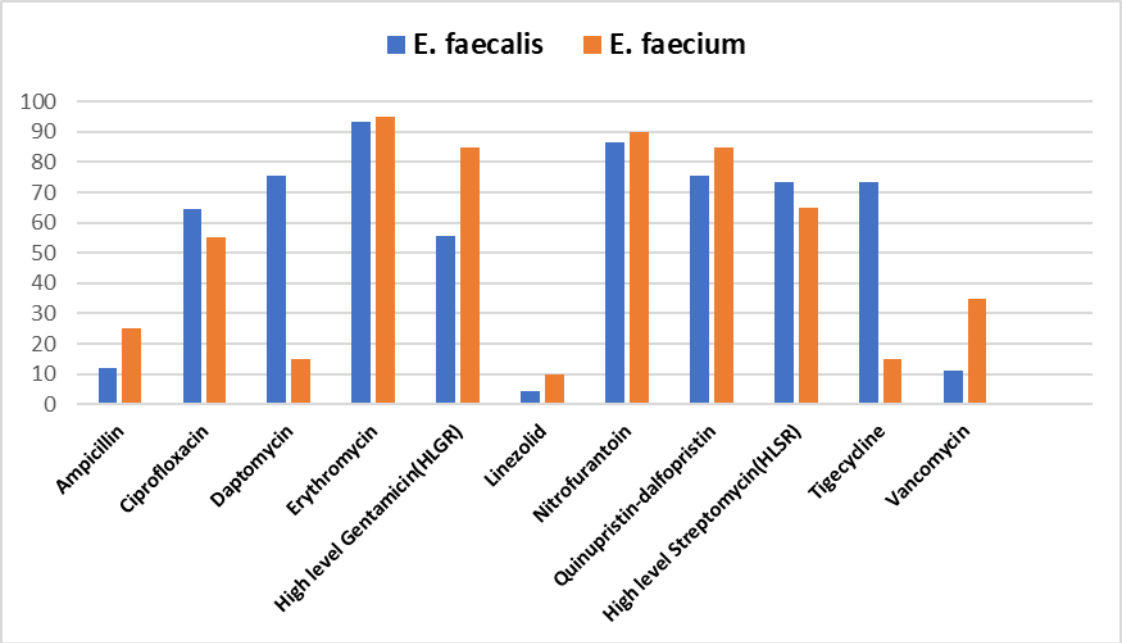

Fig.1: Resistance percentage of *E. faecalis* and *E. faecium* of the tested antibiotics

Regarding vancomycin susceptibility, 71.1%, 17.8 % and 11.1% of *E. faecalis* and 55 %, 10% and 35% of *E. faecium* were sensitive, intermediately resistant, and resistant to vancomycin respectively. For linezolid susceptibility, 73.4%, 22.2% and 4.4% of *E. faecalis* and 85%, 5% and 10% of *E. faecium* were sensitive, intermediately resistant, and resistant to linezolid with non-significant difference between both species (**Table 2**).

Vancomycin sensitive *E. faecalis* and *E. faecium* were significantly sensitive to linezolid ($p < 0.001$), about 90.6%, 100% of vancomycin sensitive were linezolid sensitive. However non-significant relation between vancomycin resistance and resistance or intermediate resistance to linezolid ($p > 0.05$) where only 40% and 28.6% of vancomycin resistant *E. faecalis* and *E. faecium* respectively were linezolid resistant (**Table 2**).

Table 2: Linezolid susceptibility in relation to vancomycin susceptibility among *E. faecalis* and *E. faecium* CIs.

MIC	Vancomycin susceptibility (n=65)								Test Z test	P value
Linezolid susceptibility (n=65)	*E. faecalis*				*E. faecium*					
	S (n=32) (71.1%)	I (n=8) (17.8%)	R (n=5) (11.1%)	Total (n=45) (69.2%)	S (n=11) (55%)	I (n=2) (10%)	R (n=7) (35%)	Total (n=20) (30.8%)		
Sensitive (n=50) (76.9%)	29 (90.6%)	2 (25.0%)	2 (40.0%)	33 (73.4%)	11 (100%)	1 (50.0%)	5 (71.4%)	17 (85.0%)	0.37 0.17 0.49 0.71	0.71[1] 0.86[2] 0.62[3] 0.48[4]
Intermediate resistant (n= 11) (16.9%)	3 (9.4%)	6 (75.0%)	1 (20.0%)	10 (22.2%)	0 (0.0%)	1 (50.0%)	0 (0.0%)	1 (5.0%)	0.37 0.17 0.18 1.35	0.71[1] 0.86[2] 0.86[3] 0.18[4]
Resistant (n= 4) (6.2%)	0 (0.0%)	0 (0.0%)	2 (40.0%)	2 (4.4%)	0 (0.0%)	0 (0.0%)	2 (28.6%)	2 (10.0%)	---- ---- 0.21 0.30	-----[1] -----[2] 0.84[3] 0.76[4]
Test (P value)	6.25 <0.001	1.50 0.13	0.0 1.0	4.22 <0.001	4.26 <0.001	1.0 0.32	1.07 0.29	4.11 <0.001		

In relation to linezolid susceptibility
1 = Comparing vancomycin sensitivity in *E. faecalis & E. faecium*
2 = Comparing vancomycin intermediate sensitivity in *E. faecalis & E. faecium*
3= Comparing vancomycin resistance in *E. faecalis & E. faecium*
4+ Comparing linezolid, sensitivity, intermediate resistance, and resistance between *E. faecalis & E. faecium*

The most frequent virulence gene in *E. faecalis* was *gelE* (48.9%) followed by *asa1* (44.4%), *esp* (33.3%), *hyl* (28.9%) and finally *cylA*(4.4%). And for *E. faecium*, the most frequent one was *asa1* (40%) followed by *gelE* (30%), *esp* (20%) and finally 5% for each of *cylA* and *hyl*. The *vanA*, *vanB, cfr* and *optrA* genes were detected in 2.2%, 6.7%, 2.2 and 2.3 of *E. faecalis* and in 30%, 5%, 0.0% and 5% of *E. faecium* CIs respectively, with non-significant difference between *E. faecalis* and *E. faecium* in different clinical specimens expect for total and blood *vanA* that was significantly higher (p= 0.004 & 0.01) in *E. faecium* (30%, 75%) than *E. faecalis* (2.2%, 5.9%) (**Table 3& fig. 2a, b& c**).

Table 3: Frequency of virulence gene, VR genes and Linezolid R genes in *E. faecalis* and *E. faecium* in different burn patient's specimens.

Specimens	Virulence genes					VR genes		Linezolid R genes	
	asa1	***gelE***	***cylA***	***esp***	***Hyl***	***Van A***	***Van B***	***Cfr***	***Optr A***
***E. faecalis* (n=45)**									
Wound (n=18) (40 %)	9 (50.0%)	8 (44.4%)	2 (11.1%)	4 (22.2%)	2 (11.1%)	0 (0.0%)	1 (5.6%)	0 (0.0%)	1 (5.6%)
Blood (n=17) (37.7%)	4 (23.5%)	11 (64.7%)	0 (0.0%)	7 (41.2%)	10 (58.8%)	1 (5.9%)	2 (11.8%)	1 (5.9%)	1 (5.9%)
Urine (n=10) (22.3%)	7 (70.0%)	3 (30.0%)	0 (0.0%)	4 (40.0%)	1 (10.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	20 (44.4%)	22 (48.9%)	2 (4.4%)	15 (33.3%)	13 (28.9%)	1 (2.2%)	3 (6.7%)	1 (2.3%)	2 (2.2%)
***E. faecium* (n=20)**									
Wound (n=10) (50%)	3 (30.0%)	4 (40.0%)	0 (0.0%)	1 (10.0%)	1 (10.0%)	2 (20.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Blood (n=4) (20%)	2 (50.0%)	0 (0.0%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	3 (75.0%)	1 (25.0%)	0 (0.0%)	1 (25.0%)
Urine (n=6) (30%)	3 (50.0%)	2 (33.3%)	1 (16.7%)	2 (33.3%)	0 (0.0%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total	8 (40.0%)	6 (30.0%)	1 (5.0%)	4 (20.0%)	1(5.0%)	6 (30.0%)	1(5.0%)	0 (0.0%)	1 (5.0%)
Test (P value)	0.63 (0.53)[1] 0.44 (0.66)[2] 0.27 (0.79)[3] 0.06 (0.95)[4]	0.17 (0.86)[1] 1.78 (0.08)[2] 0.42 (0.67)[3] 1.15 (0.25)[4]	0.33 (0.74)[1] -------[2] 0.27 (0.79)[3] 0.54 (0.59)[4]	0.29 (0.77)[1] 0.23 (0.82)[2] 0.27 (0.79)[3] 0.80 (0.43)[4]	0.55 (0.58)[1] 1.56 (0.12)[2] 27 (0.79)[3] 1.84 (0.07)[4]	1.20 (0.23)[1] 2.46 (0.01)[2] 0.27 (0.79)[3] 2.09 (0.004)[4]	0.30 (0.76)[1] 0.11 (0.90)[2] -------[3] 0.30 (0.76)[4]	-----[1] 0.81 (0.41)[2] --------[3] 0.42 (0.67)[4]	0.30 (0.76)[1] 0.23 (0.82)[2] --------[3] 0.54 (0.59)[4]

Regarding virulence gene, VR genes and Linezolid R genes
1= Comparing wound isolates between *E. faecalis* & *E. faecium*.
2 = Comparing blood isolates between *E. faecalis* & *E. faecium*.
3 = Comparing urine isolates between *E. faecalis* & *E. faecium*.
4 = Comparing total isolates between *E. faecalis* & *E. faecium*.

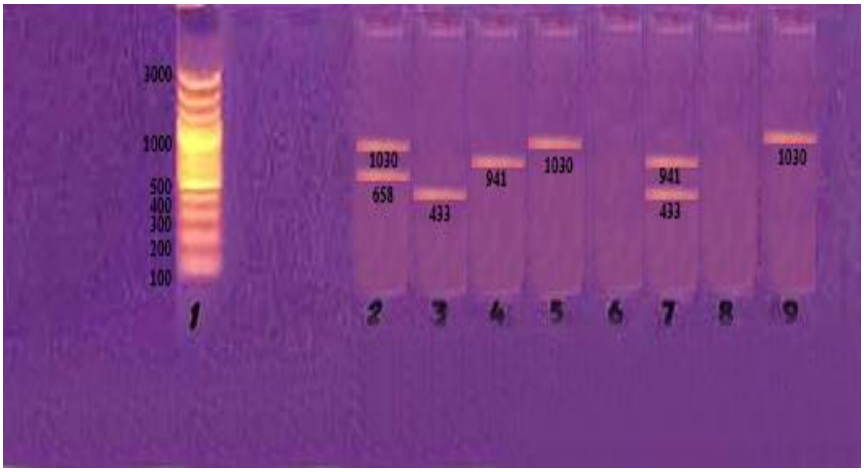

Fig. 2a: Gel electrophoresis of PCR amplification product of $ddl_{E.\ faecalis}$, $ddl_{E.\ faecium}$, *vanA* and *vanB* genes. Lane 1; DNA ladder (100-3000bp), lanes 2, 5 and 9; + ve *vanA* gene (1030bp), lanes 3 and 7; +ve *vanB* gene (433bp), lanes 4 and 7; +ve $ddl_{E.\ faecalis}$ gene (941 bp) and lane 2; +ve $ddl_{E.\ faecium}$ gene (658 bp).

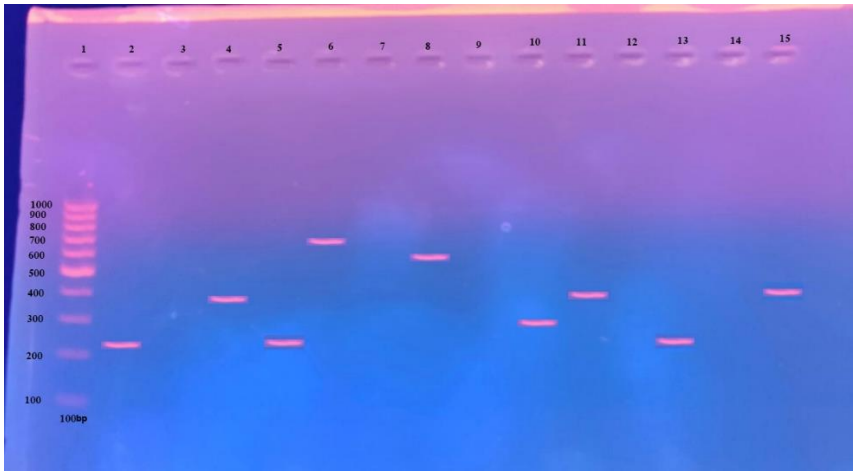

Fig.2b: Gel electrophoresis of PCR amplification product of *E. faecium* and *E. faecalis* virulence genes. Lane 1; DNA ladder (100- 1000bp). Lanes 2, 5 and 13; *gelE* gene (213bp). Lane 10; *hyl* gene (276bp). Lanes 4 and 11; *asa1*gene (375bp). Lane8; *esp* gene (510 bp) and lane 6; *cylA* gene (688bp).

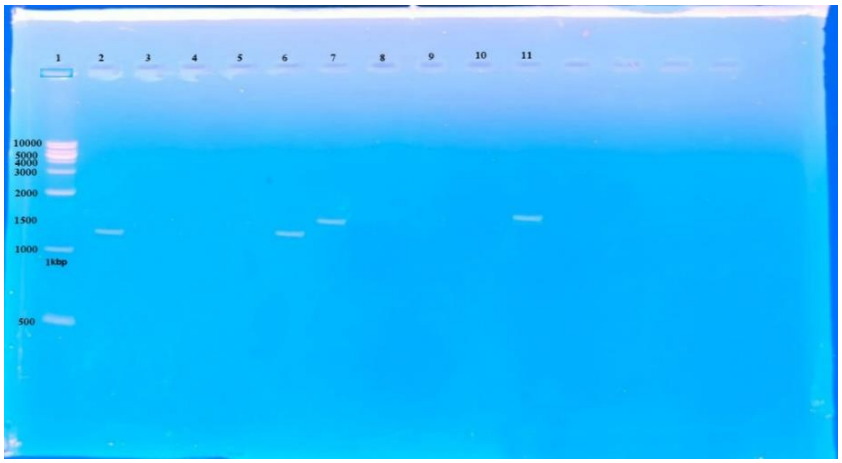

Fig. 2c: Gel electrophoresis of PCR amplification product of linezolid *cfr* and *optrA* resistance genes. Lane 1; DNA ladder (1kbp), lanes 2 and 6; + ve *cfr* gene (1320bp), lanes 7 and 11; +ve *optrA* gene (1390bp). Lanes 3,4,5, 8, 9 and 10; -ve for both genes.

There was non-significant difference in vancomycin susceptibility between *E. faecalis* & *E. faecium* (p> 0.05) except in the presence of *asa1* gene where *E. faecium* significantly resistant (62.5%) to vancomycin than *E. faecalis* (20.0%) (p value 0.009). Also, *E. faecium* that had *esp* gene showed significant vancomycin resistance (p value: 0.03) with non-significant effect on *E. faecalis*. *E. faecalis* was significantly sensitive to vancomycin (p value 0.03) although, the presence of *gelE* virulence gene, with non-significant effect *cylA*. *esp* and *hyl* genes. Also, there was non-significant effect of *cylA*, *gelE* and *hyl on E. faecium* vancomycin susceptibility (**Table 4**).

The *vanA, vanB, cfr and optrA genes* were present in 100% of vancomycin resistant *E. faecalis* like *E. faecium* except that, the *cfr* was not detected. The *van A* was significantly higher (p value 0.004) in *E. faecium* than *E. faecalis*, with non-significant difference regarding *van B*, *cfr*, and *optr A* genes (**Table 4**).

Table 4: Vancomycin-susceptibility in relation to virulence genes, vancomycin, and linezolid resistance genes among *E. faecalis* and *E. faecium* isolates.

	Virulence genes					VR genes		Linezolid R genes	
	***E. faecalis* (n=45)**								
	***asa1* (n=20) (44.5%)**	***gelE* (n=22) (48.8%)**	***CylA* (n=2) (4.5%)**	***Esp* (n=15) (33.3%)**	***Hyl* (n=13) (28.9%)**	***Van A* (n=1) (2.2%)**	***Van B* (n=3) (6.6%)**	***Cfr* (n=1) (2.2%)**	***Optr A* (n=2) (4.4%)**
Vancomycin sensitive (n=32)	15(75.0%)	15(68.2%)	0(0.0%)	7(46.7%)	5(38.5%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
Vancomycin-Intermediate (n=8)	1 (5.0%)	5(22.2%)	1(50.0%)	4(26.7%)	5(38.5%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
Vancomycin-resistant (n=5)	4 (20.0%)	2 (9.1%)	1(50.0%)	4(26.7%)	3(23.1%)	1(100%)	3(100.0%)	1(100%)	2(100%)
Test (p value)	2.85 0.004	2.11 0.03	1.0 0.32	0.0 1.0	0.78 0.43	0.0 1.0	1.63 0.10	0.0 1.0	1.0 0.32
	E. faecium (n=20)								
	***asa1* (n=8) (40%)**	***gelE* (n=6) (30%)**	***CylA* (n=1) (5%)**	***Esp* (n=4) (20%)**	***Hyl* (n=1) (1%)**	***Van A* (n=6) (30%)**	***Van B* (n=1) (5%)**	***Cfr* (n=0) (0.0%)**	***Optr A* (n=1) (5%)**
Vancomycin sensitive(n=11)	1(12.5%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Vancomycin-Intermediate (n=2)	2 (25.0%)	2 (33.3%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Vancomycin-resistant (n=7)	5 (62.5%)	3 (50.0%)	1 (100%)	3 (75.0%)	1 (100%)	6 (100%)	1 (100%)	0 (0.0%)	1 (100%)
Test (p value)	2.50 0.01	1.73 0.08	0.0 1.0	2.12 0.03	0.0 1.0	2.89 0.004	0.0 1.0	------	0.0 1.0
Test (P value)	2.6(0.009)[1] 0.87(0.38)[2] 1.73(0.08)[3]	1.79(0.07)[1] 0.0(1.0)[2] 1.72(0.07)[3]	------[1] 0.43(0.67)[2] 0.43(0.67)[3]	1.14(0.25)[1] 0.57(0.57)[2] 1.20(0.23)[3]	0.31(0.76)[1] 0.31(0.76)[2] 0.49(0.62)[3]	------[1] -------[2] ------- [3]	------[1] ------[2] -------[3]	-----[1] ------[2] --------[3]	-----[1] ------[2] -------[3]

In relation to virulence gene, VR genes and Linezolid R genes
1= Comparing vancomycin sensitivity between *E. faecalis* & *E. faecium*
2 = Comparing vancomycin intermediate resistance between *E. faecalis* & *E. faecium*
3 = Comparing vancomycin resistance between *E. faecalis* & *E. faecium*

Although *E. faecalis* had *asa1*, *gelE* and *Hyl* virulence genes, they showed significant linezolid susceptibility (p= 0.02, 0.03 & 0.02) respectively with non-significant effect of *cylA*, *esp* nor *Van A*, *Van B*, *Cfr* and *Optr A* genes. There was non-significant effect for the presence of *asa1*, *gelE*, *cylA*, *esp* and *Hyl* virulence genes nor *Van A*, *Van B*, *Cfr* and *Optr A* genes on *E. faecium* linezolid susceptibility with non-significant difference between *E. faecalis* & *E. faecium* (**Table5**).

Table 5: Linezolid susceptibility in relation to virulence genes, vancomycin, and linezolid resistance genes among *E. faecalis* and *E. faecium* isolates.

	Virulence genes					VR genes		Linezolid R genes	
	E. faecalis (n=45)								
	***asa1* (n=20)**	***gelE* (n=22)**	***cylA* (n=2)**	***Esp* (n=15)**	***Hyl* (n=13)**	***Van A* (n=1)**	***Van B* (n=3)**	***cfr* (n=1)**	***Optr A* (n=2)**
linezolid- sensitive (n=33)	**14 (70.0%)**	**15 (68.2%)**	**1 (50.0%)**	**10 (66.7%)**	**10(76.9%)**	**0(0.0%)**	**0(0.0%)**	**0(0.0%)**	**0(0.0%)**
linezolid-Intermediate/ resistant (n=12)									
4 (µg/mL): (n=7)	4(20.0%)	6 (27.3%)	1(50.0%)	2(13.3%)	0(0.0%)	0(0.0%)	1(33.3%)	0(0.0%)	0(0.0%)
8 (µg/mL): (n=2)	1(5.0%)	1(6.7%)	0(0.0%)	2(13.3%)	2(15.4%)	0(0.0%)	1(33.3%)	0(0.0%)	1(50.0%)
16 (µg/mL): (n=3)	1(5.0%)	0(0.0%)	0(0.0%)	1(6.7%)	1(7.7%)	1(100%)	1(33.3%)	1(100%)	1(50.0%)
Test (p value)	2.21 0.02	2.11 0.03	1.0 0.32	1.46 0.14	2.35 0.02	0.0 1.0	1.63 0.10	0.0 1.0	1.0 0.32
	E. faecium (n=20)								
	***asa1* (n=8)**	***gelE* (n=6)**	***cylA* (n=1)**	***Esp* (n=4)**	***Hyl* (n=1)**	***Van A* (n=6)**	***Van B* (n=1)**	***Cfr* (n=0)**	***Optr A* (n=1)**
linezolid-sensitive (n=17)	6(75.0%)	3(50.0%)	1(100%)	2(50.0%)	1(100%)	5 (83.3%)	0(0.0%)	0(0.0)	0(0.0%)
linezolid-Intermediate/ resistant (n=3)									
4 (µg/m)(n=1)	1(12.5%)	1 (16.7%)	0(0.0%)	1(25.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)	0(0.0%)
8 (µg/mL) (n=1)	1(12.5%)	1(16.7%)	0(0.0%)	1(25.0%)	0(0.0%)	0(0.0%)	1(100%)	0(0.0%)	0(0.0%)
16 (µg/m)(n=1)	0(0.0%)	1(16.7%)	0(0.0%)	0(0.0%)	0(0.0%)	1(16.7%)	0(0.0%)	0(0.0%)	1(100%)
Test (p value)	1.5 0.13	0.58 0.57	0.0 1.0	0.71 0.48	0.0 1.0	1.73 0.08	0.0 1.0	-----	0.0 1.0
Test (P value)	0.20 (0.84)[1] 0.08 (0.94)[2] 0.12 (0.91)[3] 0.48 (0.63)[4]	0.34 (0.73)[1] 0.0 (1.0)[2] 0.13 (0.90)[3] 0.71 (0.48)[4]	0.43 (0.67)[1] 0.43 (0.67)[2] ----[3] -----[4]	0.03 (0.98)[1] 0.20 (0.84)[2] 0.20 (0.84)[3] 0.73 (0.47)[4]	0.72 (0.47) -------[2] 1.06 (0.86)[3] 1.73 (0.08)[4]	0.51 (0.61)[1] ----------[2] ----------[3] 0.51 (0.62)[4]	------[1] 0.67 (0.51)[2] 0.0 (1.0)[3] 0.67 (0.51)[4]	------[1] -------[2] ---------[3] ----------[4]	------[1] -------[2] 0.42 (0.67)[3] 0.43 (0.67)[4]

1= Comparing linezolid- sensitivity between *E. faecalis* & *E. faecium*
2 = Comparing linezolid- Intermediate/ resistant (4 µg/mL) between *E. faecalis* & *E. faecium*
3 = Comparing linezolid- Intermediate/ resistant (8 µg/mL) between *E. faecalis* & *E. faecium*
4 = Comparing linezolid- Intermediate/ resistant (16 linezolid- Intermediate/ resistant (4 (µg/mL) µg/mL) between *E. faecalis* & *E. faecium*

DISCUSSION

In our study, *enterococcus* spp. was isolated from 24.1% (n=65/270) of the burn patient's different CIs. among them *E. faecalis* represented 69.2% (n=45) and *E. faecium* was 30.8% (n=20) distributed in wound, blood, and urine specimens as 40% (n=18), 37.7%(n=17) and 22.3% (n=10) for *E.faecalis* and 50%(n=10), 20%(n=4) and 30%(n=6) for *E. faecium* respectively. This approximately agrees with Shokoohizadeh et al[2], where enterococci were isolated from 16.4% (n=56), among them, 62.5% (n = 35) were recognized as *E. faecalis* and 37.5% (n = 21) as *E. faecium* distributed in wound, blood, and urine specimens as 37.5% (n = 21), 30.3% (n = 17), and 35% (n = 18), respectively. Wang et al [27], reported that *E. faecalis* is responsible for greater than 80% of enterococcal infections and *E. faecium* is progressively more reported. Said and Abdelmegeed[3] reported that, the isolated enterococci were detected in urine, blood and wound; 54.4%, 35% and 10.7% respectively. Ma et al [14] agreed that *E. faecalis* is one of the main urinary microbes in urinary tract infected patients. For Tawfick et al.[28], *enterococci* spp. were detected in 20.8% (50/240) of isolated specimens where, *E. faecium* represented 46% followed by *E. faecalis* (30%).

In our study the results towards antibiotics tested were nearly similar to other reports[3,4,14]. This study showed no association between virulence genes and different clinical samplings like previous study[2].

Our study showed that *E. faecalis* carry more virulence genes (VF) than *E. faecium.* The *asa1* and *gelE* were the highly detected ones in *E. faecalis* (44.4% and 48.9%) and *E. faecium* (40.0% and 30.0%) respectively. The *cylA* was the least one, detected in *E. faecalis* (4.4%) and in *E. faecium* (5%). That is nearly consistent with Shokoohizadeh et al [2], who documented greater presence of VF in *E. faecalis* than *E. faecium* and detected *asa1* and *gelE* in 48.5% of *E. faecalis* and in 43% of *E. faecium*. Also, Kiruthiga et al[29], documented that *gelE* was reported frequently (76.4%) in *enterococci* spp., and detected in 85.39% of *E. faecalis* and 60.78% in *E. faecium*. That differs from Vankerckhoven et al[20], where *asa1*, *gelE*, and *cylA* genes were not detected in his study on *E. faecium* isolates that agreed with the results reported by other investigators[17, 30] or detected by lower incidence as in a study done by Ravichandran et al[31]. Also, Eaton and Gasson[32] detected one *gelE*-positive *E. faecium* isolate and Elsner et al[33] detected *asa1* only in 13% of *E. faecium* CIs. Kiruthiga et al[29] documented *cylA* in 13.88%, 2.17% of *E. faecalis* and *E. faecium* respectively. Tawfick et al[28] detected *cylA* gene in 32% of enterococcus CIs. The *cylA* detected in extremely minimal rate in *E. faecium* than *E. faecalis*[34].

In our study, the *esp* gene was reported in 33.3 % of *E. faecalis* and 20% of *E. faecium*. That is lower than that by Vankerckhoven[20] and Eaton and Gasson[32], who detected *esp* gene in 65% and 80% of *E. faecium* and higher than Shankar et al[18], who documented the absence of *esp* in *E. faecium*. Kiruthiga[29], detected esp in 53.93% of *E. faecalis* that was higher than *E. faecium* (45.09%) as previously reported[35].

The *hyl* gene in our study was detected in 28.9% in *E. faecalis* and 5% in *E. faecium*, As described before, *hyl* was detected only in *E. faecium*[36]. However, a few studies have described the prevalence of hyl in both species[29].

The present study reported 11.1%, 4,4% of *E. faecalis* and 35%, 10% of *E. faecium* were resistant to vancomycin and linezolid respectively where only 40% (n=2) and 28.6%(n=2) of VR *E. faecalis* and *E. faecium* were linezolid resistant. This coincides with Said and Abdelmegeed [3] and O'Driscoll et al[37] results that showed vancomycin resistance in *E. faecium* higher than that in *E. faecalis* CIs. And slightly higher than the study by Shokoohizadeh et al [2] where no resistance to vancomycin nor linezolid were observed in *E. faecalis* isolates and only 20% of *E. faecium* exhibited resistance to vancomycin. Latest research in Iran reported that 18.8% of their enterococcal CIs. were vancomycin resisant[38]. On the other hand, Ma et al [14] found that all urinary *E. faecalis* including linezolid-intermediate/resistant CIs., were even vancomycin sensitive, denying any cross resistance between linezolid and vancomycin. Tawfik et al[28] and Klare et al[39], reported that 8 % and 9.7% of Enterococcus isolates were resistant to linezolid respectively.

Our study showed significant effect of the presence of *vanA* in *E. faecium* with non-significant effect in *E. faecalis* regarding vancomycin resistance where 20% (1/5), 60% (3/5) of VR *E. faecalis* and 85.7% (6/7), 14.3% (1/7) of VR *E. faecium* carry *vanA* and *vanB* respectively. This is in agreement with Jahansepas et al[38]. But lower than Shokoohizadeh et al[2] report regarding *vanA* and higher regarding *van B* where all *E. faecium* were resistant to vancomycin (*VREfm*) had *vanA*, while no *vanB* gene was detected in *VREfm* strains. Tawfik et al[28] reported that one *E. faecium* isolate was intermediately resistant to vancomycin had *vanB* gene.

The 1st *optrA*-carrying *E. faecium* isolate of human origin was detected in 2005 in China. It mediates resistance to oxazolidinones and phenolics[27]. In our study *optrA* gene was detected in 16.7% (2/12) of *E. faecalis* that showed intermediate resistance (MIC 8µg/mL) or resistance (MIC 16µg/mL) to linezolid and 100.0% of *E. faecium* that showed linezolid resistance (MIC 16µg/mL) with non-significant effect of it on linezolid susceptibility. the *optrA* gene was reported in 80% of the enterococci that showed linezolid resistance that is considered as the first *optrA* gene report in linezolid resistant enterococci in Egypt[3].

In this study, the *cfr* gene that mediate resistance to oxazolidinones was detected only in 2.2% *E. faecalis* with non-significant relation between *cfr* resistance gene and linezolid resistance that might explained by the low number of linezolid resistant enterococci. However, Diaz et al[26] reported the relation between *cfr* resistance gene and linezolid resistance, and various studies detected the *cfr* gene among linezolid resistant enterococci[26, 39].

Regarding enterococcal virulence gene, this study showed non-significant effect of *asa1*, *gelE*, *Hyl*, *cylA* and *esp* virulence genes on the vancomycin resistance in *E. faecalis.* However, in *E. faecium*, the occurrence of *asa1*, *esp* virulence genes was significantly related to vancomycin resistance. Also, Vankerckhoven et al [20], significantly reported the *esp* gene in a higher number (P <0.0001) of VR *E. faecalis* strains (77%) than VS *E. faecalis* strains (53%). Prior studies on the *esp* prevelance among VREF and VSEF were contradictory[30]. An equivalent allocation of the *esp* gene was found among VREF and VSEF strains as previously described[19]. Shokoohizadeh et al[2] reported *VREfm* showed several virulence factors. Tawfik et al[28] found the *hyl* gene among 16% and 17% among VREF and VSEF CIs. respectively. Rice et al[19] found the *hyl* gene only in VREF.

CONCLUSIONS

LZD resistant enterococci increasingly detected, with no significant relation between linezolid resistance and vancomycin resistance nor virulence factors.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

Acknowledgements:

I would like to appreciate the efforts of all staff members of Burn Unit, General Surgery Department, Molecular Unit, Medical Microbiology Department and Central Laboratory for their cooperation during the study.

REFERENCES

1. Arshadi M, Douraghi M, Shokoohizadeh L, Moosavian SM, Pourmand MR. High prevalence of diverse vancomycin resistance *Enterococcus faecium* isolates in clinical and environmental sources in ICU wards in southwest of Iran. Microb Pathog. 2017; 111: 212–7.
2. Shokoohizadeh L, Ekrami A, Labibzadeh M, Ali L and Alavi SM. Antimicrobial resistance patterns and virulence factors of enterococci isolates in hospitalized burn patients. BMC Res Notes. 2018; 11(1): 2-5.
3. Said HS and Abdelmegeed ES. Emergence of multidrug resistance and extensive drug resistance among enterococcal clinical isolates in Egypt. Infection and Drug Resistance. 2019; 12:1113-1125.
4. Jones ME, Draghi DC, Tornsberry C, Karlowsky JA, Sahm DF, and Wenzel RP. Emerging resistance among bacterial pathogens in the intensive care unit- a European and North American surveillance study (2000–2002). Annals of Clinical Microbiology and Antimicrobials. 2004; 3(14): 1-11.
5. Jett BD, Huycke MM, and Gilmore MS. Virulence of enterococci. Clinical Microbiology Reviews.1994; 7(4): 462–478.
6. Golob M, Pate M, Kušar D, Dermota U, Avberšek J, Bojan Papic and Zdovc I. Antimicrobial Resistance and Virulence Genes in *Enterococcus faecium* and *Enterococcus faecalis* from Humans and Retail Red Meat: BioMed Research International. 2019; 1-12.
7. Dutka-Malen S and Courvalin P. Update on glycopeptide resistance in enterococci. Antimicrob. Newsl. 1990; 7: 81-86.
8. Leclercq R, Derlot E, Duval J, and Courvalin P. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. 1988; 319:157-161.
9. Quintiliani R Jr, Evers S, and Courvalin P. The *vanB* gene confers various levels of self-transferable resistance to vancomycin in enterococci. J. Infect. Dis. 1993; 167:1220- 1223.
10. Dutka-malen S, Evers S, and Courvalin P. detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by pcr. journal of clinical microbiology. 1995; 33(1): 24-27.
11. Moosavian M, Ghadri H, Samli Z. Molecular detection of *vanA* and *vanB* genes among vancomycin-resistant enterococci in ICU hospitalized patients in Ahvaz in southwest of Iran. Infect Drug Resist. 2018; 11: 2269-2275.
12. Wilson DN, Schluenzen F, Harms JM, Starosta AL, Connell SR, Fucini P. The oxazolidinone antibiotics perturb the ribosomal peptidyl-transferase center and effect tRNA positioning. Proc Natl Acad Sci USA. 2008; 105:13339- 44.
13. Chen M, Pan H, Lou Y, et al. Epidemiological characteristics, and genetic structure of linezolid-resistant *Enterococcus faecalis*. Infect Drug Resist. 2018; 11: 2397- 2409.
14. Ma X, Zhang F, Bai B, Lin Z, Xu G, Chen Z, Sun X, Zheng J, Deng Q and Yu Z. Linezolid Resistance in *Enterococcus faecalis* Associated with Urinary Tract Infections of Patients in a Tertiary Hospitals in China: Resistance Mechanisms, Virulence, and Risk Factors. Frontiers in Public Health. 2021; 9 (570650): 1-8.
15. Galli D, Lottspeich F, and Wirth R. Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex pheromone plasmid pAD1. Mol. Microbiol.1990; 4: 895-904.
16. Su YA, Sulavik MC, He P, Makinen KK, Makinen PL, Fiedler S, Wirth R, and Clewell DB. Nucleotide sequence of the gelatinase gene (*gelE*) from *Enterococcus faecalis* subsp. Liquefaciens. Infect. Immun. 1991; 59: 415-420.
17. Coque TM, Patterson JE, Steckelberg JM, and Murray BE. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J. Infect. Dis. 1995; 171: 1223-1229.

18. Shankar V, Baghdayan AS, Huycke MM, Lindahl G, and Gilmore MS. Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. Infect. Immun. 1999; 67:193–200.
19. Rice LB, Carias L, Rudin S, Vael C, Goossens H, Konstabel C, Klare I, Nallapareddy SR, Huang W, and Murray BE. A potential virulence gene, hyl_{Efm}, predominates in *Enterococcus faecium* of clinical origin. J. Infect. Dis. 2003; 187: 508-512.
20. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D, and Goossens H. 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*. journal of clinical microbiology. 2004; 42(10): 4473–4479.
21. Willems RJ, Homan W, Top J, Santen-Verheuvel M, Tribe D, Manzioros X, Gaillard C, Vandenbroucke-Grauls CM, Mascini EM, Van Kregten E, Van Embden JD, and Bonten MJ. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. Lancet. 2001; 357: 853-855.
22. Woodford N, Soltani M, and Hardy KJ. Frequency of *esp* in *Enterococcus faecium* isolates. Lancet. 2001;358: 584.
23. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 31st ed. CLSI supplement. 2021; M100. Wayne, PA.
24. Clinical and Laboratory Standards Institute. Methods for Dilution of Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard- 31st ed. CLSI Document. 2021; M07-A10.
25. Kariyama R, Mitsuhata R, Chow JW, Clewell DB, and Kumon H. simple and reliable multiplex pcr assay for surveillance isolates of vancomycin-resistant enterococci. journal of clinical microbiology. 2000; 38(8): 3092-3095.
26. Diaz L, Kiratisin P, Mendes RE, Panesso D, Singh KV, and Ariasa CA. Transferable Plasmid-Mediated Resistance to Linezolid Due to *cfr* in a Human Clinical Isolate of *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy. 2012; 56(7): 3917- 3922.
27. Wang Y, Lv Y, Cai J, Schwarz S, Cui L, Hu Z, Zhang R, Li J, Zhao Q, He T, Wang D, Wang Z, Shen Y, Li Y, Feßler AT, Wu C, Yu H, Deng X, Xia Xand Shen J. A novel gene, *optrA*, that confers transferable resistance to oxazolidinones and phenicols and its presence in *Enterococcus faecalis* and *Enterococcus faecium* of human and animal origin. J Antimicrob Chemother. 2015; 70: 2182-2190.
28. Tawfik MM, El Menofy NG, Omran ME, Alsharony OA and Abo-Shady MA. Phenotypic and Molecular Characterization of PlasmidMediated Virulence and Antimicrobial Resistance Traits among Multidrug Resistant *Enterococcus Spp*. in Egypt. Journal of Pure and Applied Microbiology. 2020; 14(3):1649-1661.
29. Kiruthiga A, Padmavathy K, Shabana P, Naveenkumar V , Gnanadesikan S and Malaiyan J. Improved detection of *esp*, *hyl*, *asa1*, *gelE*, *cylA* virulence genes among clinical isolates of Enterococci. BMC Res Notes. 2020; 13(170):1-7.
30. Dupre I, Zanetti S, Schito AM, Fadda G, and Sechi LA. Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). J. Med. Microbiol. 2003; 52: 491- 498.
31. Ravichandran L, Sivaraman U, Pramodhini S, Srirangaraj S, Seetha KS. Prevalence of virulence factors among clinical isolates of *Enterococcus spp*. Asian J Pharm clin Res. 2016; 9(9):72–5.
32. Eaton TJ, and Gasson MJ. Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. 2001; 67:1628–1635.
33. Elsner HA, Sobottka I, Mack D, Claussen M, Laufs R, and Wirth R. Virulence factors of *Enterococcus faecalis* and *Enterococcus faecium* blood culture isolates. Eur. J. Clin. Microbiol. Infect. Dis. 2000; 19:39-42.
34. Huycke MM, Spiegel CA, Gilmore MS. Bacteremia caused by hemolytic, high-level gentamicin-resistant *Enterococcus faecalis*. Antimicrob Agents Chemother. 1991;35(8):1626-34.
35. 35.Tendolkar PM, Baghdayan AS, Shankar N. Pathogenic enterococci: new developments in the 21st century. Cell Mol Life Sci. 2003; 60(12):2622-36.
36. Dogru AK, Gencay YE, Ayaz ND. Comparison of virulence gene profles of *Enterococcus faecium* and *Enterococcus faecalis* chicken neck skin and faeces isolates. Kafkas Üniversitesi Veteriner Fakültesi Dergisi. 2010; 16:129-33.
37. O'Driscoll T, Crank CW. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. Infect Drug Resist. 2015; 8:217–230.
38. Jahansepas A, Aghazadeh M, Rezaee MA, et al. Occurrence of *Enterococcus faecalis* and

Enterococcus faecium in Various Clinical Infections: Detection of Their Drug Resistance and Virulence Determinants. Microb Drug Resist. 2018; 24(1):76-82.

39. Klare I, Fleige C, Geringer U, et al. Increased frequency of linezolid resistance among clinical *Enterococcus faecium* isolates from German hospital patients. J Glob Antimicrob Resist. 2015; 3(2):128-131.