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Antimicrobial Susceptibility and Resistance Genes Patterns in *Enterococcus* spp. Isolated from Hospitalized Patients from Northern Jordan

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Abstract

Enterococcus faecalis and *Enterococcus faecium* are normal gut microbiota in humans. They are major opportunistic nosocomial pathogens and are capable of acquiring antimicrobial resistance determinants. Determining the prevalent resistance genotypes and phenotypes among these species should enable better management of clinical infections. In this study, the prevalence of *E. faecalis* and *E. faecium* carriage among hospitalized patients from Northern Jordan was investigated via culture of peri-anal swabs on selective media coupled with biochemical testing. Antimicrobial susceptibility was assessed against 10 antimicrobial agents using the Kirby-Bauer method. The percentages of vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*) and linezolid resistance genes (*cfr*, *optrA* and *poxtA*) among the isolates were determined by two multiplex PCR assays followed by gel electrophoresis for detection of amplification products. Overall, among 271 patients sampled, 114 *E. faecalis* and 33 *E. faecium* isolates were recovered (147 total; 54.2% carriage). The highest non-susceptibility to antimicrobials was for rifampin (104/147; 70.7%) and erythromycin (96/147; 65.3%), while the highest susceptibility was for linezolid (147/147; 100%) and tigecycline (145/147; 98.6%). Overall, vancomycin and linezolid resistance genes were relatively infrequent among the isolates. *VanB* (35/147; 23.8%) was the most frequent vancomycin resistance gene, followed by *vanA* and *vanC* (26/147; 17.7% each), *vanG* (23/147; 15.6%), and *vanD* and *vanE* (15/147; 10.2% each). *OptrA* (20/147; 13.6%) was the most frequent linezolid resistance gene, followed by both *cfr* and *poxtA* (7/147; 4.8% each). Several resistance genotypes were associated with specific antimicrobial non-susceptibility patterns. Namely, each of *vanA* and *vanD* with non-susceptibility to ampicillin and teicoplanin, *optrA* with non-susceptibility to doxycycline and chloramphenicol, *vanB* with non-susceptibility to ciprofloxacin, *vanD* with non-susceptibility to chloramphenicol, and *vanG* with non-susceptibility to tigecycline. In conclusion, linezolid and tigecycline remain good treatment choices for *E. faecalis* and *E. faecium* associated infections for hospitalized patients. However, due to continuous changes in antimicrobial resistance patterns among bacteria, periodical monitoring of resistance genotypes and phenotypes should be performed.

Keywords: Vancomycin, Linezolid, antimicrobial agents, resistance genes, *Enterococcus*, carriage, nosocomial.

1. Introduction

Enterococci are Gram-positive bacteria belonging to group D streptococci. They can cope with harsh environmental conditions such as high salt concentrations and a wide temperature range (10 °C to >45 °C) (Arias and Murray, 2012). There are 83 known species among genus *Enterococcus*. Two species are common commensal organisms in the human intestines (*E. faecium* and *E. faecalis*). Other species are occasionally found in the human intestines. *E. faecium* and *E. faecalis* are major opportunistic pathogens causing nosocomial infections. The ability of enterococci to acquire resistance against several antimicrobial agents limits infection treatment choices, and increases rates of morbidity and mortality (Arias and Murray, 2012).

Resistance to antimicrobial agents among enterococci and particularly *E. faecium* and *E. faecalis*, is attributed to three factors; intrinsic resistance, tolerance, and acquired resistance (Kristich *et al.*, 2014). Intrinsic resistance is natural resistance encoded in the bacterial genome. *E. faecium* and *E. faecalis* intrinsically exhibit decreased susceptibility to β-lactams (i.e., penicillinase-resistant penicillins and cephalosporins), and low in vivo concentrations of trimethoprim-sulfamethoxazole, fluoroquinolones, clindamycin, and aminoglycosides (Hollenbeck and Rice, 2012). Acquired resistance is the ability to express resistance to virtually any antimicrobial agent either by mutations or by acquisition of resistance determinants via horizontal gene transfer (Hollenbeck and Rice, 2012). *Enterococci* exhibit acquired resistance to a wide range of antimicrobials such as β-lactams, aminoglycosides, chloramphenicol, erythromycin, tetracyclines, glycopeptides (Hollenbeck and Rice, 2012).

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Tolerance is the ability of microorganisms to survive at a slow rate of growth in presence of lethal concentrations of bactericidal agents (Brauner *et al.*, 2016). Enterococci are tolerant to bactericidal antimicrobial agents that inhibit cell wall synthesis, such as β-lactams and vancomycin (Kristich *et al.*, 2014).

Glycopeptide antimicrobial agents such as vancomycin and teicoplanin were the drugs of choice for treatment of infections by Gram-positive organisms such as enterococci. The rise of resistance to glycopeptide antimicrobials limited treatment options to a few antimicrobial agents such as tetracyclines (tigecycline), lipopeptides (daptomycin), and oxazolidinones (linezolid and tedizolid) (Bender *et al.*, 2018). Overall, eight glycopeptide resistance genes have been described to date among vancomycin resistant enterococci (VRE); *vanA, vanB, vanC1, vanC2, vanC3, vanD, vanE, vanG* (Bhatt *et al.*, 2015).

Linezolid, the first member of oxazolidinones, was approved in 2000 in the USA to treat VRE and other resistant Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant pneumococci (Zahedi Bialvaei *et al.*, 2017). Linezolid acts by blocking bacterial protein synthesis, leading to either death or growth inhibition. Unlike other protein inhibitors that interfere with the elongation step of protein synthesis, linezolid blocks the initiation step by binding to the A site on the bacterial 23S rRNA of the 50S subunit (Hashemian *et al.*, 2018). The first linezolid resistance case was reported in 2001, just one year following linezolid's approval for clinical use in the USA. Since then, several studies have reported the appearance of linezolid resistance among enterococci worldwide (Wada *et al.*, 2024).

Several mechanisms mediating resistance to linezolid have been described to date among enterococci. These include point mutations in 23S rRNA genes (e.g., T2500A, G2447T, C2534T, G2215A, and T2504A), mutations in ribosomal proteins L3 and/or L4, the acquisition of the plasmid-borne ribosomal methyltransferase gene *cfr* or its variant *cfr(B)*, and the acquisition of the novel genes *optrA* and *poxtA* encoding for an ABC-F transporter protein (Deshpande *et al.*, 2015, Bi *et al.*, 2018, Hao *et al.*, 2019). The first transferable oxazolidinone resistance genes to emerge in enterococci are *cfr* and *cfr(B)*. The products of these two genes methylate carbon number 8 of the A2503 nucleotide located in the linezolid binding site on the bacterial 23S rRNA. This prevents binding of linezolid to the A site on the bacterial ribosome, thus preventing its antibacterial activity (LaMarre *et al.*, 2013). The *optrA* and *poxtA* oxazolidinone resistance genes encode ribosomal protection proteins of the ABC-F family (Hao *et al.*, 2019). *OptrA* and *poxtA* confer resistance to several antimicrobials including oxazolidinones, macrolides, tetracyclines, streptogramins, lincosamides, ketolides, pleuromutilins, and phenicols (Antonelli *et al.*, 2018).

Tigecycline, which was approved in 2006, is the first member of the glycylcycline class of antimicrobial agents. It is a broad-spectrum antimicrobial agent that is active against Gram-positive bacteria such as penicillin-resistant *Streptococcus pneumoniae*, MRSA, methicillin-resistant *Staphylococcus epidermidis* (MRSE), and VRE. Tigecycline is structurally related to minocycline and tetracycline. However, the presence of an *N,N,*-dimethylglycylamido group at the 9th position of the minocycline molecule makes tigecycline effective against tetracycline-resistant bacteria and expands its spectrum of activity. Tigecycline is a bacteriostatic antimicrobial agent that inhibits bacterial protein synthesis by binding to the 30S ribosome. This binding inhibits the elongation stage of protein synthesis (Yaghoubi *et al.*, 2022).

Tigecycline resistance occurs due to drug efflux from the bacterial cell, or ribosomal protection via decreased binding of tigecycline to the bacterial 30S ribosome. In tigecycline resistant enterococci, the *tet(L)* gene is associated with antibiotic efflux, while *tet(M)* and *rpsJ* genes are associated with ribosomal protection. Acquisition of *tet(L)* which encodes for Major Facilitator Superfamily (MFS)-type efflux pump decreases enterococcal susceptibility to tigecycline by mediating drug efflux outside the cell. Acquisition of *tet(M)* or a mutation in *rpsJ* (encoding the S10 protein of the 30S ribosomal subunit) results in a decrease in enterococcal susceptibility to tigecycline by preventing the binding of tigecycline to the bacterial 30S ribosome (Bender *et al.*, 2018).

The surveillance of vancomycin, linezolid, and tigecycline resistant enterococcal isolates and the associated resistance genes have not been investigated in Jordan. This is the first study to report on the prevalence of vancomycin, linezolid, and tigecycline non-susceptibility in enterococci, namely *E. faecium* and *E. faecalis* from patients admitted to several hospitals in Northern Jordan. The prevalence of several vancomycin resistance genes (*vanA, vanB, vanC1, vanC2, vanD, vanE, vanG*) and linezolid resistance genes (*cfr, optrA* and *poxtA*) was also determined and correlated to the non-susceptibility phenotypes.

2. Materials and Methods

2.1. Collection of Isolates

Study was approved by the IRB committee of Jordan University of Science and Technology (Approval# 32/127/2019; Date: 10/10/2019). All participants or the legal guardian provided informed written consent before study enrollment. Peri-anal swabs were collected from in-patients at hospitals in Northern Jordan (King Abdullah University Hospital, Princess Rahma Hospital, Princess Badea'a Hospital, Al-Ramtha Hospital, Al-Yarmouk Governmental Hospital, and Princess Basma Hospital). The swabs were inoculated on an *Enterococcus*-selective medium; bile esculin azide agar plates (Sigma-Aldrich, St. Louis, Missouri, United States). The plates were incubated aerobically at 35ºC for 18-24 hours. *Enterococcus* colonies appeared small and transparent and were surrounded by blackened media due to hydrolysis of esculin to esculetin (a black compound) and glucose.

2.2. Identification of Isolates

Isolates that hydrolyzed esculin to esculetin were identified as enterococci. To further investigate the isolates to the species level, one colony from the primary culture was inoculated in two broth media tubes (phenol red sorbitol broth and phenol red raffinose broth). The original color of these two broth media is reddish-orange. The fermentation of the sugar (sorbitol or raffinose) produces acids which converts the original color to yellow. Isolates

with a positive result for sorbitol fermentation were identified as *Enterococcus faecalis*, while isolates with a positive result for raffinose fermentation were identified as *Enterococcus faecium*. Isolates that were negative for both sorbitol and raffinose fermentation were identified as non-*faecalis* and non-*faecium* enterococci and were excluded from further analyses. All isolates were grown in LB broth (Himedia, Maharashtra, India) overnight. Next, 0.7 mL of the broth was mixed with 0.35 mL of sterile 50% glycerol in a 2 mL cryotube, and the tube was stored at -80ºC.

2.3. Antimicrobial Susceptibility Testing

Muller Hinton Agar (MHA) (Himedia, Maharashtra, India) was inoculated from the frozen cultures and incubated aerobically at 35ºC for 18-24 hours. Next, three to four well isolated colonies having the same morphology were transferred using a sterile disposable loop, from the MHA plate into a sterile tube containing 2.0 mL sterile normal saline. The colonies were re-suspended by shaking the loop in the normal saline solution to create a suspension with a turbidity equivalent to 0.5 McFarland. The suspension was used to inoculate MHA plates for antimicrobial susceptibility testing according to the Kirby-Bauer disk diffusion method. A swab was used to inoculate MHA in 3 directions. The inoculated plates were covered and left for 15 minutes in the upright position before placement of antibiotic disks at least 2 cm apart utilizing a sterile lancet. The antimicrobial agents used were ampicillin (10 µg), doxycycline (30 µg), teicoplanin (30 µg), linezolid (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), vancomycin (30 µg), rifampin (5 µg), chloramphenicol (30 µg), and tigecycline (15 µg). All antibiotic disks were obtained from Oxoid (Basingstoke, Hampshire, United Kingdom).

Antimicrobial susceptibility testing was done using two MHA plates per isolate (5 antibiotic disks per plate). The plates were incubated at 37ºC for 18 hours. The diameter of the inhibition zones was measured in mm and was interpreted according to CLSI (2023) guidelines. *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* 19433, and *Escherichia coli* ATCC 25922 were used as controls during antimicrobial susceptibility testing.

2.4. DNA Extraction

For DNA extraction, the G-spin ™ genomic DNA extraction kit (Catalogue #17121, iNtRON Biotechnology, Inc., Korea), was used as described by the manufacturer. One colony from each sample was inoculated in Muller Hinton broth (Himedia, Maharashtra, India) overnight (18-24 hours). Next, 1 mL of the broth was transferred to a 1.5 mL Eppendorf tube and centrifuged for 1 min at 13,000 rpm, and the supernatant was discarded. To improve yield of the extracted DNA, the previous step was repeated 3 times (by resuspending the pellet in 1 mL of broth each time). After obtaining the bacterial pellet, 50 µL of Pre-Buffer and 3 µL of Lysozyme solution were added, mixed well, and the tube incubated for 15 min at 37°C. Next, 250 µL of G-Buffer solution was added, mixed, and incubated at 65°C for 15 min. After cell lysis, 250 µL of the Binding Buffer was added and mixed well by pipetting (at least 10 times). The cell lysates were loaded on columns containing silica membranes and were centrifuged for 1 min at 13000 rpm. Next, 2 washing steps were done (by adding washing buffer A, centrifugation, removing the solution then adding washing buffer B, centrifugation and discarding the solution). Before the last step (the elution step) was done, the column was centrifuged for 1 min at 13,000 rpm and the column was placed in a new sterile Eppendorf tube. Next, 100 µL of Elution Buffer was directly placed onto the membrane of the column, incubated at room temperature for 1 min and centrifuged for 1 min at 13,000 rpm. DNA samples were stored at -18°C until used for PCR. *Enterococcus faecalis* ATCC 29212 and *Enterococcus faecalis* 19433, were used as controls during PCR detection of resistance genes.

2.5. Multiplex PCR

The protocol of the multiplex PCR for detection of glycopeptide (vancomycin) resistance genes (*vanA, vanB, vanC1, vanC2, vanD, vanE, vanG*) and primer sequences were obtained from previously published work (Bhatt *et al.*, 2015). Each PCR consisted of 10 µL of template DNA, 1.25 µL of each primer (each at 10 µmol/L), 0.4 µL Taq DNA Polymerase (250 U/mL), 5 µL of PCR buffer (10X) with $MgCl_2$ (15 mM), 0.25 µL dNTPs (10 mM), and nuclease free water up to 50 µL. The primer sequences used are shown in Table 1. PCR thermal cycling was done using initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. PCR without the DNA template was used as negative control.

Table 1. Primers used for multiplex PCR to detect vancomycin resistance genes.

Primer	Sequence (5' to 3')	Size of the product
VanA(forward)	GGGAAAACGACAATTGC	732 bp
VanA (reverse)	GTACAATGCGGCCGTTA	
VanB (forward)	ACGGAATGGGAAGCCGA	647 bp
VanB (reverse)	TGCACCCGATTTCGTTC	
VanC (forward)**	ATGGATTGGTAYTKGTAT*	815/827 bp
VanC (reverse)**	TAGCGGGAGTGMCYMGTAA*	
VanD (forward)	TGTGGGATGCGATATTCAA	500 bp
VanD (reverse)	TGCAGCCAAGTATCCGGTAA	
VanE (forward)	TGTGGGATCGGAGCTGCAG	430 bp
VanE (reverse)	ATAGTTTAGCTGGTAAC	
VanG (forward)	CGGCATCCGCTGTTTTTGA	941 bp
VanG (reverse)	GAACGATAGACCAATGCCTT	

*K = G or T; M = A or C; Y= C or T **detect VanC1 and VanC2

Primer sequences were obtained from a previous study (Bhatt *et al.*, 2015).

The protocol for the multiplex PCR detection of linezolid resistance genes (*cfr*, *optrA* and *poxtA*) was obtained from previously published work (Bender *et al.*, 2019). Primer sequences were obtained from previously published works as shown in table 2 (Kehrenberg and Schwarz, 2006, Brenciani *et al.*, 2016, Bender *et al.*, 2019). Each PCR had 5-10 ng of DNA template, 0.1 µM of each primer, 12.5 µL 2X DreamTaq Master Mix, and nuclease free water up to 25 µL. PCR thermal cycling was done using initial denaturation at 96°C for 2 min, followed by 30 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. PCR without the DNA template was used as negative control.

Table 2. Primers used in the multiplex PCR to detect linezolid resistance genes.

Primer	Sequence (5' to 3')	Size of the product
Cfr (forward)	TGAAGTATAAAGCAGGTTGGGAGTCA	746 bp
Cfr (reverse)	ACCATATAATTGACCACAAGCAGC	
OptrA (forward)	TACTTGATGAACCTACTAACCA	422 bp
OptrA (reverse)	CCTTGAACTACTGATTCTCGG	
PoxtA (forward)	AAAGCTACCCATAAAATATC	533 bp
PoxtA (reverse)	TCATCAAGCTGTTCGAGTTC	

Primer sequences were obtained from previous studies (Kehrenberg and Schwarz, 2006, Brenciani *et al*., 2016, Bender *et al*., 2019).

2.6. Gel Electrophoresis

Products from PCR were separated on 2% agarose gels containing redsafe dye. Ten microliters of 100 bp ladder (iNtRON Biotechnology, Inc., Korea) were loaded in the first well to allow identification of band sizes. Ten microliters of PCR products were loaded in the remaining wells. Electrophoresis was performed using constant voltage (90 V) for 1 h. Bands on the gel were visualized using a UV transilluminator provided with a gel documentation system using the Quantity One software (Biorad, USA). Band sizes were determined by comparison with the 100 bp DNA ladder.

2.7. Statistical Analysis

Statistical analysis and tables were done using SPSS software (version 26, IBM, Armonk, NY, USA). Charts were created using Microsoft Excel (Microsoft, Redmond, Washington, United States). Pearson Chi-Square test was used to determine the significance of the observed frequencies. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Isolates Identification and Distribution

Overall, 271 anal swabs were collected from patients admitted to several hospitals in Northern Jordan. Among them 114 *E. faecalis* and 33 *E. faecium* isolates were identified based on the hydrolysis of esculin in bile esculin azide agar and whether the isolate fermented sorbitol or raffinose sugar. The overall carriage of *Enterococcus* species was 54.2% (147/271). Most samples were from King Abdullah University Hospital. The distribution of *E. faecalis* or *E. faecium* isolates according to patient sex and hospital is shown in Table 3.

Table 3. Distribution of *Enterococcus* spp. isolates according to sex and hospital.

		Isolates recovered				Total	P value
		E. faecalis		*E. faecium*			
		n	%	n	%		
Hospital	Badea'a	6	85.7	1	14.3	7	0.360
	KAUH	64	71.9	25	28.1	89	
	Rahma	30	88.2	4	11.8	34	
	Al-Ramtha	5	83.3	1	16.7	6	
	Yarmouk	9	81.8	2	18.2	11	
Sex	Female	66	86.8	10	13.2	76	0.005
	Male	48	67.6	23	32.4	71	

KAUH: King Abdulla University Hospital.

Anal swabs were collected from different hospital departments (internal medicine, general surgery, gynecology and obstetrics, pediatrics, psychiatry, neurology, ear, nose and throat, orthopedic, ophthalmology; Data not shown). Most swabs were collected from internal medicine, general surgery, pediatrics and gynecology and obstetrics departments. Isolates were obtained from all age groups; from 18 days to 81 years. The mean age was 18.8 ± 22.0 years.

3.2. Antimicrobial Susceptibility Profile

The antimicrobial susceptibility of *E. faecalis* or *E. faecium* isolates is presented in Figure 1. The results are classified into susceptible and non-susceptible groups (intermediately susceptible + resistant). The highest non-susceptibility was against rifampin (70.7%), erythromycin (65.3%), and doxycycline (56.5%). All isolates were susceptible to linezolid. Following linezolid, the highest susceptibility was against tigecycline (98.6%), teicoplanin (98%), and vancomycin (98%).

No significant differences in antimicrobial susceptibility results were observed according to species for all antimicrobials except ampicillin and doxycycline. Susceptibility of *E. faecalis* to ampicillin was significantly higher compared to *E. faecium*. On the other hand, *E. faecalis* had significantly lower susceptibility to doxycycline compared to *E. faecium*.

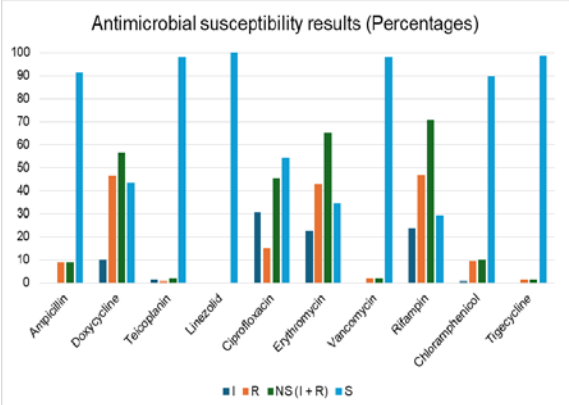

Figure 1. Antimicrobial susceptibility of isolates. All data represent percentages. Antimicrobial susceptibility of the isolates was determined using the Kirby-Bauer method. Interpretation of inhibition zones (if any) was based on CLSI 2023 guidelines. I: intermediate susceptibility. NS: non-susceptible. R: resistant. S: susceptible.

3.3. Resistance Genes

The frequencies of vancomycin and linezolid resistance genes among the isolates were determined by multiplex PCR. Table 4 shows the frequencies of the detected resistance genes among the isolates. *VanB* was the most frequent at 23.8% (35/147), followed by *vanA* and *vanC* (26/147; 17.7% each), *vanG* (23/147; 15.6%), *optrA* (20/147; 13.6%), *vanD* and *vanE* (15/147; 10.2% each), and finally *cfr* and *poxtA* (7/147; 4.8% each) (Figure 2).

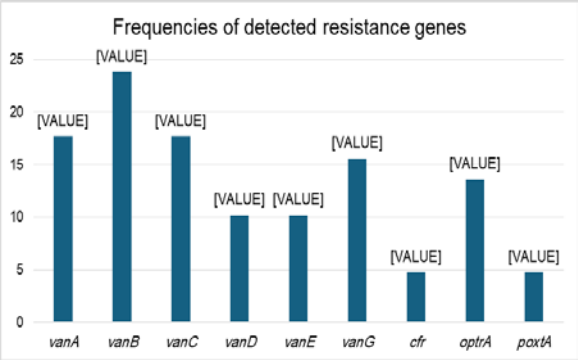

Figure 2. Frequencies of the resistance genes among the isolates. Resistance genes were amplified using two multiplex PCR assays followed by separation on agarose gels and detection.

The association of resistance genes with antimicrobial susceptibility profile was also investigated (Tables 4 and 5). The results show a significant association between each of *vanA* and *vanD* and non-susceptibility to ampicillin and teicoplanin. *OptrA* was associated with non-susceptibility to doxycycline and chloramphenicol. *VanB* was associated with non-susceptibility to ciprofloxacin. *VanD* was associated with non-susceptibility to chloramphenicol. *VanG* was associated with non-susceptibility to tigecycline. Interestingly, *cfr* was associated with susceptibility to ciprofloxacin.

Table 4. The association of resistance genes with antimicrobial susceptibility profile of the isolates (part 1).

		Ampicillin					Doxycycline					Teicoplanin					Linezolid			Ciprofloxacin				
		NS		S			NS		S			NS		S			S			NS		S		
Resistance Gene		n	%	n	%	P value	n	%	n	%	P value	n	%	n	%	P value	n	%	P value	n	%	n	%	P value
vanA	N	8	6.6	113	93.4	0.040	67	55.4	54	44.6	0.565	1	0.8	120	99.2	0.025	121	100	-	53	43.8	68	56.2	0.351
	P	5	19.2	21	80.8		16	61.5	10	38.5		2	7.7	24	92.3		26	100		14	53.8	12	46.2	
vanB	N	11	9.8	101	90.2	0.455	63	56.3	49	43.8	0.926	3	2.7	109	97.3	0.328	112	100	-	45	40.2	67	59.8	0.019
	P	2	5.7	33	94.3		20	57.1	15	42.9		0	0.0	35	100		35	100		22	62.9	13	37.1	
vanC	N	9	7.4	112	92.6	0.195	71	58.7	50	41.3	0.243	2	1.7	119	98.3	0.473	121	100	-	53	43.8	68	56.2	0.351
	P	4	15.4	22	84.6		12	46.2	14	53.8		1	3.8	25	96.2		26	100		14	53.8	12	46.2	
vanD	N	5	3.8	127	96.2	0.000	72	54.5	60	45.5	0.164	1	0.8	131	99.2	0.001	132	100	-	60	45.5	72	54.5	0.929
	P	8	53.3	7	46.7		11	73.3	4	26.7		2	13.3	13	86.7		15	100		7	46.7	8	53.3	
vanE	N	13	9.8	119	90.2	0.203	75	56.8	57	43.2	0.796	3	2.3	129	97.7	0.555	132	100	-	59	44.7	73	55.3	0.525
	P	0	0.0	15	100		8	53.3	7	46.7		0	0.0	15	100		15	100		8	53.3	7	46.7	
vanG	N	10	8.1	114	91.9	0.440	71	57.3	53	42.7	0.652	3	2.4	121	97.6	0.451	124	100	-	57	46.0	67	54.0	0.826
	P	3	13.0	20	87.0		12	52.2	11	47.8		0	0.0	23	100		23	100		10	43.5	13	56.5	
cfr	N	11	7.9	129	92.1	0.060	80	57.1	60	42.9	0.457	3	2.1	137	97.9	0.696	140	100	-	67	47.9	73	52.1	0.013* I
	P	2	28.6	5	71.4		3	42.9	4	57.1		0	0.0	7	100		7	100		0	0.0	7	100	
optrA	N	9	7.1	118	92.9	0.059	67	52.8	60	47.2	0.022	3	2.4	124	97.6	0.487	127	100	-	61	48.0	66	52.0	0.132
	P	4	20.0	16	80.0		16	80.0	4	20.0		0	0.0	20	100		20	100		6	30.0	14	70.0	
poxtA	N	11	7.9	129	92.1	0.060	80	57.1	60	42.9	0.457	3	2.1	137	97.9	0.696	140	100	-	64	45.7	76	54.3	0.882
	P	2	28.6	5	71.4		3	42.9	4	57.1		0	0.0	7	100		7	100		3	42.9	4	57.1	

I: Inverse association. N: negative. NS: non-susceptible. P: positive. S: susceptible. P < 0.05 are significant (Chi-square test).

Table 5. The association of resistance genes with antimicrobial susceptibility profile of the isolates (part 2).

Resistance Gene		Erythromycin					Vancomycin					Rifampin					Chloramphenicol					Tigecycline				
		NS		S			NS		S			NS		S			NS		S			NS		S		
		n	%	n	%	P value	n	%	n	%	P value	n	%	n	%	P value	n	%	n	%	P value	n	%	n	%	P value
vanA	N	75	62.0	46	38.0	0.068	2	1.7	119	98.3	0.473	84	69.4	37	30.6	0.446	11	9.1	110	90.9	0.336	2	1.7	119	98.3	0.509
	P	21	80.8	5	19.2		1	3.8	25	96.2		20	76.9	6	23.1		4	15.4	22	84.6		0	0.0	26	100	
vanB	N	74	66.1	38	33.9	0.727	3	2.7	109	97.3	0.328	77	68.8	35	31.3	0.341	12	10.7	100	89.3	0.715	1	0.9	111	99.1	0.381
	P	22	62.9	13	37.1		0	0.0	35	100		27	77.1	8	22.9		3	8.6	32	91.4		1	2.9	34	97.1	
vanC	N	77	63.6	44	36.4	0.359	2	1.7	119	98.3	0.473	85	70.2	36	29.8	0.774	13	10.7	108	89.3	0.641	2	1.7	119	98.3	0.509
	P	19	73.1	7	26.9		1	3.8	25	96.2		19	73.1	7	26.9		2	7.7	24	92.3		0	0.0	26	100	
vanD	N	84	63.6	48	36.4	0.207	2	1.5	130	98.5	0.181	96	72.7	36	27.3	0.118	11	8.3	121	91.7	0.026	2	1.5	130	98.5	0.631
	P	12	80.0	3	20.0		1	6.7	14	93.3		8	53.3	7	46.7		4	26.7	11	73.3		0	0.0	15	100	
vanE	N	88	66.7	44	33.3	0.304	3	2.3	129	97.7	0.555	91	68.9	41	31.1	0.153	13	9.8	119	90.2	0.673	1	0.8	131	99.2	0.061
	P	8	53.3	7	46.7		0	0.0	15	100		13	86.7	2	13.3		2	13.3	13	86.7		1	6.7	14	93.3	
vanG	N	79	63.7	45	36.3	0.345	3	2.4	121	97.6	0.451	90	72.6	34	27.4	0.257	13	10.5	111	89.5	0.795	0	0.0	124	100	0.001
	P	17	73.9	6	26.1		0	0.0	23	100		14	60.9	9	39.1		2	8.7	21	91.3		2	8.7	21	91.3	
cfr	N	93	66.4	47	33.6	0.201	3	2.1	137	97.9	0.696	100	71.4	40	28.6	0.417	14	10.0	126	90.0	0.715	2	1.4	138	98.6	0.750
	P	3	42.9	4	57.1		0	0.0	7	100		4	57.1	3	42.9		1	14.3	6	85.7		0	0.0	7	100	
optrA	N	84	66.1	43	33.9	0.592	3	2.4	124	97.6	0.487	94	74.0	33	26.0	0.028	9	7.1	118	92.9	0.002	1	0.8	126	99.2	0.131
	P	12	60.0	8	40.0		0	0.0	20	100		10	50.0	10	50.0		6	30.0	14	70.0		1	5.0	19	95.0	
poxtA	N	91	65.0	49	35.0	0.727	3	2.1	137	97.9	0.696	99	70.7	41	29.3	0.968	15	10.7	125	89.3	0.361	2	1.4	138	98.6	0.750
	P	5	71.4	2	28.6		0	0.0	7	100		5	71.4	2	28.6		0	0.0	7	100		0	0.0	7	100	

N: negative. NS: non-susceptible. P: positive. S: susceptible. $P < 0.05$ are significant (Chi-square test).

4. Discussion

E. faecium and *E. faecalis* are members of the gut microbiota of the human intestines. Yet they are considered major opportunistic microorganisms associated frequently with nosocomial infections. The intrinsically low susceptibility of enterococci to several antimicrobial drugs and their ability to express and acquire resistance against several antimicrobial agents limits treatment choices and leads to increased morbidity and mortality (Arias and Murray, 2012).

In this study, *E. faecalis* and *E. faecium* isolates were recovered from peri-anal swabs from patients admitted to hospitals in Northern Jordan. Antimicrobial susceptibility to 10 antimicrobial agents and several resistance genes were investigated. This epidemiological data should enable better management of infections and should enable comparison with countries in the MENA and worldwide.

Similar to reports from Oman, Italy, Iran, and Jordan, the percentage of *E. faecalis* isolates was higher than that for *E. faecium* (77.6% vs. 22.4%, respectively. Regarding patient's sex, *E. faecalis* was significantly more frequent among females. While *E. faecium* was significantly more frequent among males. This is in agreement with a study from Kenya; *E. faecalis* was isolated more frequently from females (56.7%) than males. While 6 of the 7 *E. faecium* isolates were from males (Georges *et al.*, 2022).

In our study, a low percentage (5.3%) of *E. faecalis* isolates were non-susceptible to ampicillin. This is lower than that from Italy, Sweden, Turkey, China, Korea, Nigeria, Uganda, Kuwait, Indonesia, Israel and India. On the other hand, Australia and USA had the lowest non-susceptibility percentage to ampicillin (0%), whereas India had the highest non-susceptibility to ampicillin (74.1%) (Guan *et al.*, 2024).

Teicoplanin non-susceptibility in *E. faecalis* was very low (0.9%). This is in line with percentages from Cuba, Turkey, and Poland. On the other hand, Australia, Lebanon, Sweden and Germany had 0% teicoplanin non-susceptibility. While, Taiwan and Hungary had 81% and 80% non-susceptibility, respectively (Guan *et al.*, 2024).

All *Enterococcus* isolates were sensitive to linezolid. This is in agreement with *E. faecalis* susceptibility results to linezolid in Taiwan, Australia, and Germany. In contrast, high non-susceptibility against linezolid was reported in Turkey at 10% (Guan *et al.*, 2024).

Ciprofloxacin non-susceptibility in *E. faecalis* was moderately high (47.4%) compared to other countries. Several countries had lower non-susceptibility percentages; Cuba, Brazil, Lebanon, Poland, Nigeria, Romania, Turkey, China, Iran, Hong Kong, Uganda, Indonesia, Australia. In contrast, Taiwan had high non-susceptibility to ciprofloxacin at 83% (Guan *et al.*, 2024).

Erythromycin non-susceptibility in *E. faecalis* was relatively high at 64%, which is comparable to percentages from China and Lebanon. Cuba, Brazil, Italy, Germany, Egypt, Nigeria, Kuwait, Lebanon,

Uganda, Iran, and Australia, had lower non-susceptibility percentages than our study. In contrast, the USA, Algeria, Turkey, India, China, and South Korea had higher non-susceptibility percentages, with South Korea having the highest percentage at 91% (Guan *et al.*, 2024).

Vancomycin non-susceptibility in *E. faecalis* was low at 1.8%, which is similar to percentages from Poland, Turkey, China, and Romania. Some countries had lower non-susceptibility percentages; Australia, South Korea, Lebanon, Sweden, Cuba, Germany, and Algeria, while other countries exhibited higher vancomycin non-susceptibility; USA, Taiwan, India, Indonesia, Iran, Pakistan, Kuwait, Egypt, Nigeria, Israel, Hungary, Italy, and Brazil. Among these, Taiwan had the highest vancomycin non-susceptibility (99%) (Guan *et al.*, 2024).

Rifampin non-susceptibility in *E. faecalis* was high at 71.1%. Other countries had lower non-susceptibility percentages; Egypt, Lebanon, Cuba, Turkey, and Iran, while other countries had higher vancomycin non-susceptibility; Taiwan, South Korea, and Italy, with South Korea having the highest percentage (96%) (Guan *et al.*, 2024).

Chloramphenicol non-susceptibility in *E. faecalis* was relatively low at 12.3%. This percentage is similar to that from South Korea. Some countries had lower non-susceptibility percentages; Egypt and Sweden, while other countries had higher rifampin non-susceptibility; Hong Kong, Iran, China, India, Kuwait, Lebanon, Algeria, Italy, Brazil, and Cuba, with Italy having the highest percentage (73%) (Guan *et al.*, 2024).

Tigecycline non-susceptibility in *E. faecalis* was very low at 0.9%, which is similar to that from Italy. Other countries such as Turkey, Iran, Hungary, China, Poland, and USA reported 0% tigecycline non-susceptibility (Guan *et al.*, 2024).

Doxycycline non-susceptibility in *E. faecalis* was relatively high at 64%. Countries such as India, Italy, China, and Pakistan, reported lower non-susceptibility percentages, while countries such as Germany and Iran had relatively comparable non-susceptibility percentages of 68.9% and 73.6%, respectively (Guan *et al.*, 2024).

Among the tested antimicrobials, rifampin, erythromycin, doxycycline, and ciprofloxacin, had the highest non-susceptibility in *E. faecalis*. Therefore, these antimicrobials may not be good choices for the treatment of infections caused by *E. faecalis*. This finding agrees with studies worldwide (Guan *et al.*, 2024). On the other hand, *E. faecalis* had high susceptibility to tigecycline, teicoplanin, linezolid, and vancomycin. Therefore, these antimicrobials may be considered when dealing with *E. faecalis* infections, which is in agreement with studies worldwide (Guan *et al.*, 2024).

Unlike Jordan, wealthy countries that have an advanced health care system and strict strategies for the use of antimicrobials for managing infections (such as Australia, Sweden, and Poland) had lower *E. faecalis* non-susceptibility percentages than our study (Guan *et al.*, 2024). In contrast, countries in Southeast Asia such as Taiwan and South Korea, and Italy, among other countries, had higher *E. faecalis* non-susceptibility percentages compared to our study. This may be attributed to limited access to effective antimicrobials, unregulated administration of antimicrobials, incomplete antibiotic treatment due to financial constraints, practices of healthcare professionals, improper antimicrobial use, incomplete antibiotic supply chain, poor hygiene, insufficient education, and absence of a comprehensive antimicrobial stewardship program. In addition, specifically to Southeast Asian countries, they are popular producers of livestock and poultry, which serve as sources for the spread of antimicrobial resistance among bacteria (Guan *et al.*, 2024).

E. faecium is considered one of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens and is capable of demonstrating multi-drug resistance (Miller and Arias, 2024).

Similar to other studies, *E. faecium* non-susceptibility percentages to ampicillin, teicoplanin, erythromycin, vancomycin, and tigecycline, were higher than that in *E. faecalis* (insignificantly higher except for ampicillin). Chloramphenicol, rifampin, and ciprofloxacin non-susceptibility percentages in *E. faecium* were insignificantly lower than that in *E. faecalis*, and this could be explained by the small sample size (n=33 for *E. faecium*). *E. faecium* exhibited significantly lower non-susceptibility against doxycycline compared to *E. faecalis* (30.3%;10/33 vs. 64.0%;73/114), respectively, and this agrees with a study from Canada, in which the non-susceptibility to doxycycline was lower for *E. faecium* (39.5%) than that for *E. faecalis* (67.8%) (Kim *et al.*, 2024).

The lowest non-susceptibility percentages in *E. faecium* were against linezolid and tigecycline at 0%, followed by vancomycin and chloramphenicol with a non-susceptibility percentage of 3% for each, and this agrees with a worldwide review study in which the lowest non-susceptibility percentages were against linezolid and tigecycline. Thus, linezolid and tigecycline are considered drugs of choice for treating *E. faecium* infections in addition to *E. faecalis* infections (Jabbari Shiadeh *et al.*, 2019).

E. faecium isolates had a low vancomycin non-susceptibility percentage of 3% (1/33), and this was similar to non-susceptibility percentages from Mexico, Tunisia, Algeria, Turkey, Bangladesh, Bulgaria, Sweden, Norway, Finland, France, Belgium, Denmark, Iceland. This low percentage is lower than the mean worldwide percentage of 10%. The lowest vancomycin non-susceptibility in *E. faecium* was recorded in the Netherlands with a percentage of 0.1%. In contrast, other countries had higher non-susceptibility percentages such as India, Taiwan, Spain, United Kingdom, Poland, Egypt, Saudi Arabia, Israel, Cyprus, Italy, Germany, Portugal, USA, Chile, Brazil, Colombia, Australia, Japan, Iran, and Ireland, with Brazil having the highest non-susceptibility at 67% (Jabbari Shiadeh *et al.*, 2019).

E. faecium isolates in our study exhibited lower non-susceptibility percentages to several antimicrobials (ampicillin, teicoplanin, linezolid, vancomycin, ciprofloxacin, chloramphenicol, erythromycin, tigecycline), than those worldwide. However, *E. faecium* non-susceptibility against teicoplanin and vancomycin was lower in Africa, and was higher in America, Europe, Southeast Asia, Western pacific, and the Eastern Mediterranean, than that in our study. Furthermore, *E.*

faecium non-susceptibility against linezolid and ampicillin in our study was lower than that of all WHO regions (Jabbari Shiadeh *et al.*, 2019). Differences in the percentages of non-susceptibility among regions could be attributed to several factors, including the prevalence of resistant strains and resistance genes, socioeconomic differences, health-care practices, the use and the misuse of antimicrobials, among others.

The non-susceptibility percentages to antimicrobials in our study were mostly similar to those of previous studies. Non-susceptibility percentages in our study compared to previous studies from Jordan respectively were as follows: (2% vs. 0%) to teicoplanin, (10.2% vs. 0%) to chloramphenicol, (2% vs. 0-4%) to vancomycin, (8.8% vs. 0-15%) to ampicillin, (45.6% vs. 0-28.6%) to ciprofloxacin, (65.3% vs. 37.5%) to erythromycin, (1.4% vs. 0.9%), to tigecycline, (0% vs. 1.5%) to linezolid (Al-Tamimi *et al.*, 2022, Jordan Ministry of Health, 2023).

Regarding *van* genes, studies have shown that *vanA* was the most frequent resistance gene among *E. faecalis* and *E. faecium.* In addition, most or all VRE were positive for *vanA*. For example, 54 out of 59 VRE (91.5% of VRE) isolated from hospitalized patients in Iran were positive for *vanA*. While none were positive for *vanB* (Moosavian *et al.,* 2018). This was not consistent with our findings in which 35 out of 147 (23.8%) isolates were positive for *vanB* gene, and 26 out of 147 (17.7%) isolates were positive for each of *vanA* and *vanC*. Interestingly, a study from Australia showed that among 331 rectal specimens, 58 (17.5%) were VRE containing *vanB*, but none had *vanA*, and this was explained by the endemic spread of *vanB* among VRE in that region. *VanB* unlike *vanA*, is located on the bacterial chromosome. Thus, the likelihood of its transmission is lower than that of *vanA* (Moosavian *et al.*, 2018).

A study from Algeria demonstrated that the high non-susceptibility levels observed in strains from the ICU were associated with *vanA*. Co-existence of vanA/vanC was identified in 24.4% of *Enterococcus* spp. In surgical wards, *vanB* and *vanC* were the most frequent among isolates and were associated with intermediate non-susceptibility levels. In contrast, *vanA* was not detected among surgical ward strains (Zerrouki *et al.*, 2021).

In our study, 8 (5.4%) *Enterococcus* isolates had co-existence of vanA/vanC. Three out of 147 isolates were non-susceptible to vancomycin (2 *E. faecalis* isolates and 1 *E. faecium* isolate). The vancomycin resistant *E. faecium* isolate was positive for *vanA, vanC*, and *vanD*, while, both vancomycin resistant *E. faecalis* isolates were negative to all *van* genes. There was no statistically significant association between vancomycin non-susceptibility and *van* genes, likely due to the low frequency of vancomycin non-susceptible isolates.

Co-existence of *vanA/vanB* was reported in previous studies. A study from Iran showed that among 181 *Enterococcus* isolates, 38 (46.9%) carried *vanA*, 21 (25.9%) carried *vanB*, and 18 (22.2%) carried both *vanA* and *vanB*, while *vanC* was not detected (Madanipour *et al.*, 2017). In our study, 10 isolates demonstrated co-existence of *vanA*/*vanB* (6.8%).

VanC is recognized for its role in intrinsic resistance among certain *Enterococcus* species such as *E. gallinarum* and *E. casseliflavus*. However, its prevalence in clinically significant strains such as *E. faecalis* and *E. faecium* is limited (Sun *et al.*, 2014). Interestingly, 17.7% of our isolates harbored *vanC*. A study conducted in 13 hospitals in Greece showed that *vanC* VRE was the most prevalent, representing 57% of VRE isolates, followed by *vanA*, then *vanB* genotypes. Another study from Switzerland showed that the percentage of the *vanC* VRE genotype was 98%. This is in contrast to the USA and North Africa, where the *vanC* genotype was rare compared to the dominant *vanA* and *vanB* genotypes (Ahmed and Baptiste, 2018).

A study of *vanD* gene clusters in *E. faecium*, reported that although *vanD*-carrying strains were isolated sporadically, the prevalence of enterococci harboring *vanD* was increasing over time. The low *vanD* prevalence is likely due to its localization on large non-transferable chromosomal mobile genetic elements. A high percentage of hospitalized patients (26.7%-43.8%) demonstrated *vanD* carriage among gut anaerobic microflora but not enterococci (Sassi *et al.*, 2018). A study from the Netherlands showed that *vanD* was identified in 27.8% of patients but was not associated with vancomycin non-susceptibility (Flipse *et al.*, 2019). Similarly, in our study, the percentage of isolates carrying *vanD* was 10.2%, and none of the isolates were resistant to vancomycin.

E. faecalis and *E. faecium* strains harboring *vanG* are rare. The first documented clinical isolate of vancomycin resistant *E. faecium* containing a *vanG* gene was in 2016 in France (Sassi *et al.*, 2018). In our study, the percentage of *vanG* positive isolates was 15.6%. However, this gene was not associated with vancomycin non-susceptibility. *VanE* VRE isolates were first reported in 1999 and were associated with low level non-susceptibility to vancomycin (Fines *et al.*, 1999). *VanE* VRE isolates were then reported worldwide; e.g., USA, Canada, and Norway (Al Rubaye *et al.*, 2024). *VanE* was present among 10.2% of our isolates. However, all *vanE*-positive isolates were susceptible to vancomycin.

Enterococci positive for *vanA* are often not only resistant vancomycin but also to other antimicrobials, including teicoplanin and ampicillin (Ono *et al.*, 2005, Moosavian *et al.*, 2018). Similarly, in our study, *vanA* was associated with ampicillin and teicoplanin non-susceptibility.

OptrA is associated with non-susceptibility to oxazolidinone and phenicols. Thus, it contributes to decreased susceptibility to chloramphenicol and florfenicol (Wang *et al.*, 2015). Furthermore, *optrA* positive strains also demonstrated high levels of non-susceptibility to tetracycline (Xie *et al.*, 2025). This was in line with our findings which showed that *optrA* was associated with non-susceptibility to chloramphenicol and doxycycline.

To the best of our knowledge, the association of *vanD* with chloramphenicol non-susceptibility has not been previously reported. In addition, the association between *vanG* and tigecycline non-susceptibility and between *vanB* and ciprofloxacin non-susceptibility has not been previously reported. In our study, *optrA* was encountered in 20 isolates (13.6 %), and both *cfr* and *poxtA* were encountered in 7 isolates (4.8 %). *Cfr*, *optrA* and *poxtA* genes were associated with oxazolidinone non-susceptibility, like tedizolid and linezolid (Schwarz *et al.*, 2021), but in our study all isolates were susceptible to

linezolid. Interestingly, there was a significant association between *cfr* and ciprofloxacin susceptibility. However, due to low frequency of this gene, this requires confirmation using a larger sample size.

Overall, the frequencies of resistance genes among enterococci in our study demonstrated similarities and differences when compared to those from other regions. This may be attributed to differences in the prevalent strains and resistance genes, the type of samples and detection methods, the antimicrobials used, the implementation of antibiotic stewardship programs, and the socioeconomic characteristics of populations, among other factors.

5. Conclusions

The highest non-susceptibility for the study isolates was observed against rifampin (70.7%) and erythromycin (65.3%), while the highest susceptibility was for linezolid (100%) and tigecycline (98.6%). Vancomycin and linezolid resistance genes were relatively infrequent among the isolates. *VanB* (23.8%) was the most frequent vancomycin resistance gene, followed by *vanA* and *vanC* (17.7% each). *OptrA* (13.6%) was the most frequent linezolid resistance gene, followed by both *cfr* and *poxtA* at 4.8% each. Several resistance genotypes were associated with specific antimicrobial non-susceptibility patterns.

Future studies should use a larger sample size that is more representative of the Jordanian population to better characterize the prevalence of resistance genotypes and phenotypes in Jordan. Prevalence of other resistance genes, such as tigecycline resistance genes (*tet(L)* and *tet(M)*), could also be investigated. Furthermore, in conjunction with the Kirby-Bauer method, the broth microdilution assay could be used to determine the minimum inhibitory concentrations of the antimicrobial agents.

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