Emergence of multidrug resistance and extensive drug resistance among enterococcal clinical isolates in Egypt

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Introduction: Enterococci commonly inhabit the gastrointestinal tract of both human and animals; however, they have emerged as a leading cause of several infections with substantial morbidity and mortality. Their ability to acquire resistance combined with intrinsic resistance to various antimicrobials makes treatment of enterococcal infections challenging.

Materials and methods: The aim of the study was to evaluate the antimicrobial resistance pattern, and assess the prevalence of multidrug resistance (MDR) and extensive drug resistance (XDR) among enterococcal isolates, collected from different clinical sources, in Mansoura University Hospitals, Egypt.

Results: Antibiotic sensitivity testing revealed elevated levels of resistance among enterococcal clinical isolates (N=103). All E. faecium (N=32) and 74.6% of E. faecalis isolates (N=71) were MDR, while two E. faecalis and four E. faecium isolates were XDR. High level gentamicin resistance was detected in 79.6%, most of them carried the aac(6')-Ie-aph(2')-Ia gene. High level streptomycin resistance was seen in 36.9%, of which 52.6% carried the ant(6')-Ia gene. Resistance to macrolides and lincosamides were mediated by ermB (92.2%) and msrA/B (42.7%). tetK, tetL, and tetM genes were detected among tetracyclines resistant isolates. Resistance to vancomycin was detected in 15.5%, where vanB and vanC, gene clusters were detected in VRE isolates. Ten isolates (9.7%) were resistant to linezolid, eight of which harbored the optrA gene. Vancomycin and linezolid resistant enterococci were more likely to exhibit strong/moderate biofilm formation than vancomycin and linezolid sensitive ones.

Conclusion: Elevated levels of resistance to different classes of antimicrobial agents and emergence of MDR and XDR strains pose a major threat with limited therapeutic options for infections caused by this emerging pathogen.

Keywords: enterococci, antibiotic resistance, biofilm formation, RAPD typing, MDR enterococci, XDR enterococci, VRE, linezolid resistant enterococci

Introduction

Enterococci are Gram-positive, facultative-anaerobic cocci that are persistent in nature and can survive under adverse environmental conditions for prolonged periods of time.1 They are considered commensal organisms and members of the healthy intestinal microbiota of humans and animals.1,2 However, in the last two decades, they have emerged as one of the leading causes of several hospital-acquired and community-acquired infections with substantial morbidity and mortality.3 The most common infections caused by enterococci are urinary tract infections (UTIs), bacteremia, peritonitis, cholecystitis, meningitis, wound infections, endocarditis, and neonatal infections.3 Enterococci are considered the second etiologic agent of UTIs and the...
third of nosocomial bacteremia.1–3 Although E. faecalis is not considered as a member of the healthy oral microbiota, it has been reported in common dental diseases as dental caries and periodontitis.4,6

E. faecalis and E. faecium are the most commonly reported enterococcal species, counting for up to 90% of enterococcal infections, while other enterococcal species are rarely reported to cause human infections.2,7 Being equipped with an array of virulence determinants and capability to survive in hospital setting makes infections difficult to manage.1,2 Antimicrobial therapy of infections caused by enterococci is problematic because of its intrinsic reduced susceptibility to several frequently used antimicrobial agents including: aminoglycosides (except for high level resistance), clindamycin, cephalosporins, and trimethoprim/sulfamethoxazole.8,9 Moreover, acquired resistance through lateral gene transfer to other available antimicrobial agents, including: beta-lactams, aminoglycosides (high level resistance), macrolides, glycopeptides, and oxazolidinones, makes it more challenging.10 Vancomycin resistant enterococci (VRE) have been reported as a leading cause of outbreaks of hospital-acquired infections and in ICU-hospitalized patients.11,12 Linezolid was the drug of choice for management of infections caused by VRE; however, linezolid resistant enterococci have emerged and are reported.13,14

Clinicians may face very limited treatment options as a result of selection and spread of multidrug-resistant (MDR) and extensively drug resistant (XDR) strains.10,15 Magiorakos et al16 have defined MDR as non-susceptibility to at least one agent in three or more antimicrobial classes, while XDR has been defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial classes. Recently, enterococci have been ascribed in biofilm-associated infections of medical devices.17 The ability of enterococci to form a biofilm amplifies the difficulty of treatment due to increased antimicrobial resistance in the biofilm.18–20 Therefore, antimicrobial susceptibility testing to all available antimicrobial agents is exceptionally elemental for deciding the proper treatment of enterococcal infections.

The aim of this study is to evaluate the antimicrobial resistance pattern and assess the prevalence of MDR and XDR among enterococcal isolates collected from different clinical sources in Mansoura University Hospitals, Egypt between January and August 2017. Identification of enterococcal isolates was based on standard biochemical tests,21,22 then confirmed by a multiplex PCR system.23,24

Antimicrobial susceptibility testing

The antimicrobial susceptibility profile of enterococcal isolates was determined using a disc diffusion method.25 The inhibition zones were interpreted according to the recommendations of the Clinical and Laboratory Standard Institute.26 Susceptibility to different classes of antimicrobial agents was tested, including: ampicillin (10 µg), ampicillin/sulbactam (20 µg/10 µg), Amoxicillin (20 µg), amoxicillin/clavulanic acid (20 µg/10 µg), Imipenem (10 µg), Meropenem (10 µg), vancomycin (30 µg), clindamycin (2 µg), erythromycin (15 µg), clarithromycin (15 µg), azithromycin (15 µg), ciprofloxacin (5 µg), Levofloxacin (5 µg), tetracycline (30 µg), doxycycline (30 µg), and Linezolid (30 µg) (Oxoid, Basingstoke, UK).

The minimum inhibitory concentration (MIC) for vancomycin and linezolid was also determined by standard broth microdilution method, as per Clinical & Laboratory Standards Institute (CLSI) guidelines.26 High-level gentamicin resistance (HLGR, 500 µg/mL) and high-level streptomycin resistance (HLSR, 1000 µg/mL) were determined according to CLSI guidelines.26

Biofilm assay

A quantitative assay of biofilm formation capacity of enterococcal isolates was carried out using a polystyrene microtiter plate assay, as described previously.27,28

Molecular assays

Multiplex PCR analysis of vancomycin-resistant enterococcus species

Total genomic DNA was prepared as described previously.29 Multiplex PCR was used for the detection of vancomycin resistance determinants, including: vanA, vanB, vanC, and vanC29 genes, besides primers for the detection of the ddl genes of E. faecium and E. faecalis and of 16S rRNA as a PCR control (Table 1), as described previously.23,24

Detection of antimicrobial resistance encoding genes by PCR

The prevalence of resistance determinants to different classes of antimicrobial agents among enterococcal clinical isolates was examined. Genes encoding aminoglycosides,30,31 tetracyclines,32–34 macrolide,35 vancomycin,23,24 and linezolid36,37 resistance were detected by PCR. Primer pairs used and expected amplicon sizes are listed in Table 1.

Materials and methods

Bacterial isolates

Enterococcal isolates (N=103) were recovered from different clinical sources in Mansoura university Hospitals,
All enterococcal isolates were genotyped by RAPD-PCR, as described previously.38,39 RAPD patterns were analyzed using gelJ software.40 A similarity matrix was estimated using Dice’s coefficient, and a dendrogram was created based on the unweighted-pair group method with arithmetic averages (UPGMA).41 Enterococcal isolates with a similarity coefficient ≥85% were considered as the same genotype/clonal.

### Statistical analysis
Correlation between the capacity of enterococcal clinical isolates for biofilm formation, clinical source of collection, and resistance to different classes of antimicrobial agents were evaluated using Fisher’s exact test or chi-squared test,

**Table 1** List of primers used in this study

| Target gene | Primer pair sequence | Product size (bp) | Annealing temp (°C) | Reference |
|-------------|----------------------|------------------|---------------------|-----------|
| *rrs* (16S rRNA) | GGATTAGATACCCCTGGTATAAC | 320 | 54 | 24 |
| *ddl* _E. faecalis_ | ATCAAGTCAGTTAGTCT | 941 | 54 | 24 |
| *ddl* _E. faecium_ | TTGGGCAACCCAGGTTACCC | 658 | 54 | 24 |
| *vanA* | CATGAATAGAAATATGCTAATAA | 1,030 | 54 | 24 |
| *vanB* | AAGCTATGCAAAGGCGCATG | 536 | 54 | 23 |
| *vanC1* | GGATCAAGAAACCTC | 822 | 54 | 24 |
| *vanC2/C3* | CCGGGAAGATGGGCAGTAT | 484 | 54 | 24 |
| *aac(6’)-Ie-aph(2’’)-Ia* | CCAAGAGCAATAGGCGATA | 200 | 52 | 31 |
| *ant(6’)-Ia* | CACTATCTAACACCACTACCG | 597 | 52 | 30 |
| *blaZ* | ACCGACCCTCTCGTCTTCG | 173 | 50 | 31 |
| *tet(K)* | TTTATGTTGTTAGTACTGAA | 370 | 52 | 34 |
| *tet(L)* | GTGATGCTGCTATTTCC | 715 | 52 | 34 |
| *tet(M)* | TGAAMRWWAGGCCCCCTAA | 170 | 52 | 32 |
| *ermA* | TCCTACAGTCTGCTTACAC | 644 | 52 | 35 |
| *ermB* | GTGGATGATTTATTAATATATTAG | 639 | 52 | 35 |
| *ermC* | GCTAATTGTTTAAATCGTCAA | 642 | 52 | 35 |
| *msrA/B* | CCAAATGGTGTTAGAAGACAATCT | 400 | 52 | 35 |
| *cfr* | TGATATGTTGATTCTCTCCGACC | 1,320 | 52 | 36 |
| *optrA* | ATTATCCTCTCCAGGATTGCC | 1,395 | 55 | 37 |
| *int-Tn* | GCGTTCGTTGATCTTCTC | 1,046 | 50 | 33 |
| *xis-Tn* | GACGCTTCTGCTGCTTCT | 194 | 50 | 33 |
where a $P$-value $<$ 0.05 was considered statistically significant. Data were analyzed using the SPSS software (version 20.0; SPSS, Chicago, IL, USA).

**Results**

During the period of study, 103 enterococcal isolates were collected from Mansoura University Hospitals, Egypt from different clinical sources including: urine (56 samples, 54.4%), blood (36 samples, 35%), and wounds (11 samples, 10.7%). All isolates were identified as enterococcal species typically and genotypically. Seventy-one isolates (68.9%) were identified as *E. faecium*, while 32 isolates (31.1%) were identified as *E. faecalis*.

**Antimicrobials susceptibility of enterococcal isolates**

Among the tested enterococcal isolates, resistance to β-lactam antibiotics was distributed as follows: ampicillin (51.5% R), ampicillin/sulbactam (50.5% R, 1% I), amoxicillin and amoxicillin/clavulanic acid (47.6% R, 3.9% I). There was significant difference in resistance to β-lactam antibiotics between different enterococcal species ($P$<0.001) (Table S1). All *E. faecium* isolates (N=32) were resistant to ampicillin and β-lactam/β-lactamase inhibitor combinations, while only 29.6% of *E. faecalis* isolates (N=71) were resistant (Table 2). The *blaZ* gene was not detected in any enterococcal isolate. *E. faecalis* resistance to imipenem was (21.1% R), and meropenem was (26.8% R, 4.2% I).

Screening of high-level resistance to aminoglycosides has indicated that 82 isolates (79.6%) showed HLGR, while 38 isolates (36.9%) showed HLSR (Table 2). Among the isolates that showed HLGR, 75 isolates (91.5%) carried the *aac(6')-Ie-aph(2' ')Ia* gene (Figure S1). Among the isolates that showed HLSR, 20 isolates (52.6%) carried the *ant(6')-Ia* gene. No significant difference was observed in prevalence of HLGR between both enterococcal species; however, *E. faecium* isolates showed a higher rate of HLSR than *E. faecalis* isolates ($P$<0.001) (Table S1).

Resistance to macrolides was distributed as follows: erythromycin (91.3% R, 8.7% I), azithromycin (74.6% R, 1.4% I), and clarithromycin (85.9% R, 0% I). All isolates were resistant to clindamycin (Table 2). Among the genes encoding macrolide resistance, *ermB* was detected in 95 isolates (92.2%), while *msrA/B* was detected in 44 isolates (42.7%) (Figure S1). Both *ermB* and *msrA/B* genes were detected in 40 isolates (38.8%). *ermA* and *ermC* were not detected in any enterococcal isolate (Figures 1 and 2).

Resistance to tetracycline was (93.2% R, 5.8% I), while resistance to doxycycline was (55.3% R, 31.1% I) (Table 2). *E. faecium* isolates showed a higher rate of resistance to doxycycline than *E. faecalis* isolates ($P$=0.016) (Table S1). The *tetK* gene was detected in 62 isolates (60.2%), the *tetL*

### Table 2 Antibiotic susceptibility testing of enterococcal clinical isolates

| Antimicrobial agent | *E. faecalis*, 71 (68.9%) | *E. faecium*, 32 (31.1%) | Total, 103 (100%) |
|---------------------|---------------------------|---------------------------|------------------|
|                     | R (%) | I (%) | S (%) | R (%) | I (%) | S (%) | R (%) | I (%) | S (%) |
| Amoxicillin         | 21 (29.6) | 0 (0) | 50 (70.4) | 32 (100) | 0 (0) | 0 (0) | 53 (51.5) | 0 (0) | 50 (48.5) |
| Amoxicillin/sulbactam | 21 (29.6) | 0 (0) | 50 (70.4) | 31 (96.9) | 1 (3.1) | 0 (0) | 52 (50.5) | 1 (1) | 50 (48.5) |
| Amoxicillin         | 19 (26.8) | 2 (2.8) | 50 (70.4) | 30 (93.8) | 2 (6.3) | 0 (0) | 49 (47.6) | 4 (3.9) | 50 (48.5) |
| Amoxicillin/clavulanic acid | 19 (26.8) | 2 (2.8) | 50 (70.4) | 30 (93.8) | 2 (6.3) | 0 (0) | 49 (47.6) | 4 (3.9) | 50 (48.5) |
| Imipenem            | 15 (21.1) | 0 (0) | 56 (78.9) | 30 (93.8) | 2 (6.3) | 0 (0) | 45 (43.7) | 2 (1.9) | 56 (54.4) |
| Meropenem           | 19 (26.8) | 3 (4.2) | 49 (69) | 31 (96.9) | 1 (3.1) | 0 (0) | 50 (48.5) | 4 (3.9) | 49 (47.6) |
| HLGR                | 57 (80.3) | 0 (0) | 14 (19.7) | 25 (78.1) | 0 (0) | 7 (21.9) | 82 (79.6) | 0 (0) | 21 (20.4) |
| HLSR                | 17 (24) | 0 (0) | 54 (76.1) | 21 (65.6) | 0 (0) | 11 (34.4) | 38 (36.9) | 0 (0) | 65 (63.1) |
| Erythromycin        | 66 (93) | 5 (7) | 0 (0) | 28 (87.5) | 4 (12.5) | 0 (0) | 94 (91.3) | 9 (8.7) | 0 (0) |
| Clarithromycin      | 40 (88.9) | 0 (0) | 5 (11.1) | 21 (80.8) | 0 (0) | 5 (19.2) | 61 (59.5) | 0 (0) | 30 (30.5) |
| Azithromycin        | 35 (77.8) | 1 (2.2) | 9 (20) | 18 (69.2) | 0 (0) | 8 (30.8) | 53 (74.6) | 1 (1.4) | 17 (24) |
| Clindamycin         | 71 (100) | 0 (0) | 0 (0) | 32 (100) | 0 (0) | 0 (0) | 103 (100) | 0 (0) | 0 (0) |
| Ciprofloxacine      | 60 (84.5) | 4 (5.6) | 7 (9.9) | 24 (75) | 0 (0) | 8 (25) | 84 (81.6) | 4 (3.9) | 15 (14.5) |
| Levofloxacine       | 63 (88.7) | 1 (1.4) | 7 (9.9) | 29 (90.6) | 1 (3.1) | 2 (6.3) | 92 (89.3) | 2 (2) | 9 (8.7) |
| Tetracycline        | 66 (93) | 4 (5.6) | 1 (1.4) | 30 (93.8) | 2 (6.3) | 0 (0) | 96 (93.2) | 6 (5.8) | 1 (1) |
| Doxycycline         | 33 (46.5) | 25 (35.2) | 13 (18.3) | 24 (75) | 7 (21.9) | 1 (3.1) | 57 (55.3) | 32 (31.1) | 14 (13.6) |
| Vancomycin          | 1 (1.4) | 4 (5.6) | 66 (93) | 6 (18.8) | 5 (15.6) | 21 (65.6) | 7 (6.8) | 9 (8.7) | 87 (84.5) |
| Linezolid           | 4 (5.6) | 0 (0) | 67 (94.4) | 6 (18.8) | 0 (0) | 26 (81.3) | 10 (9.7) | 0 (0) | 93 (90.3) |

**Note:** Susceptibility for clarithromycin and azithromycin was performed on 71 isolates only. **Abbreviations:** HLGR, high-level gentamicin resistance; HLSR, high-level streptomycin resistance.


Figure 1 Genotyping of E. faecalis clinical isolates using RAPD-PCR method.

Notes: Dendrogram was created using RAPD-PCR patterns of E. faecalis clinical isolates. Similarity clustering analysis was carried out using UPGMA and Dice coefficient. The dashed line is hypothetical, indicating 85% similarity. Antibiotic susceptibility, resistance determinants, and biofilm formation capacity among E. faecalis clinical isolates were reported. Antibiotic susceptibility: Black cell: Resistant, Gray cell: Intermediate, White cell: Sensitive. Biofilm formation: White cell: Biofilm Non-producer, Light gray cell: Weak Biofilm Producer, Dark gray cell: Moderate Biofilm Producer, Black cell: Strong Biofilm Producer. Resistance determinants: Black cell: Resistance gene detected, White cell: Resistance gene not detected.

Abbreviations: AM, Ampicillin; AMC, Amoxicillin/clavulanic acid; AX, Amoxicillin; AZM, Azithromycin; CIP, Ciprofloxacin; CLR, Clarithromycin; DA, Clindamycin; DO, Doxycycline; E, Erythromycin; HLG, High Level Gentamicin Resistance; HLSR, High Level Streptomycin Resistance; IPM, Imipenem; LEV, Levofloxacin; L2D, Linezolid; MEM, Meropenem; ND, Not Determined; RAPD-PCR, random amplified polymorphic DNA-PCR; SAM, Ampicillin/Sulbactam; TE, Tetracycline; UPGMA, unweighted-pair group method with arithmetic averages; VA, Vancomycin.
Figure 2 Genotyping of *E. faecium* clinical isolates using RAPD-PCR method.

**Notes:** Dendrogram was created using RAPD-PCR patterns of *E. faecium* clinical isolates. Similarity clustering analysis was carried out using UPGMA and Dice coefficient. The dashed line is hypothetical, indicating 85% similarity. Antibiotic susceptibility, resistance determinants, and biofilm formation capacity among *E. faecalis* clinical isolates were reported. Antibiotic susceptibility: Black cell: Resistant, Gray cell: Intermediate, White cell: Sensitive. Biofilm formation: White cell: Biofilm Non-producer, Light gray cell: Weak Biofilm Producer, Dark gray cell: Moderate Biofilm Producer. Resistance determinants: Black cell: Resistance gene detected, White cell: Resistance gene not detected.

**Abbreviations:** AM, Ampicillin; AMC, Amoxicillin/clavulanic acid; AX, Amoxicillin; AZM, Azithromycin; CIP, Ciprofloxacin; CLR, Clarithromycin; DA, Clindamycin; DO, Doxycline; E, Erythromycin; HLGR, High Level Gentamicin Resistance; HLSR, High Level Streptomycin Resistance; IPM, Imipenem; LEV, Levofloxacin; LZD, Linezolid; MEM, Meropenem; ND, Not Determined; RAPD-PCR, random amplified polymorphic DNA-PCR; SAM, Ampicillin/Sulbactam; TE, Tetracycline; UPGMA, unweighted-pair group method with arithmetic averages; VA, Vancomycin.

In the study, genes were detected in the clinical isolates as follows:

- *tetK*-71 isolates (68.9%)
- *tetL*-99 isolates (96.1%)
- *tetM*-37 isolates (35.9%)
- *vanA*-55 isolates (53.4%)

Determination of MIC by broth microdilution method has indicated that seven isolates (6.8%) were resistant to vancomycin (MIC ≥ 32 µg/mL), while nine isolates (8.7%) were intermediately resistant (MIC = 8–16 µg/mL) (Table 2).

- Ten isolates (9.7%) were resistant to linezolid (MIC ≥ 8 µg/mL). Among linezolid resistant strains, eight isolates carried the *optrA* gene (Figure S1), while the *cfr* gene was not detected in any isolate. Prevalence of quinolone resistance among enterococcal isolates was (81.6% R, 3.9% I) for ciprofloxacin and (89.3% R, 2% I) for levofloxacin (Table 2).

**Biofilm formation among enterococcal isolates**

The capacity of enterococci isolated from different clinical sources to form a biofilm was evaluated by polystyrene microtiter plate assay, as previously described, and divided into strong, moderate, weak biofilm producers, and non-biofilm producers. The ability to form a biofilm was detected in almost all clinical isolates examined (97 isolates,
of antimicrobials has indicated that vancomycin-resistant isolates were more likely to exhibit a strong or moderate biofilm formation capacity than vancomycin-sensitive isolates (P=0.034) (Table S3). Moreover, all linezolid-resistant strains exhibited strong or moderate biofilm formation capacity (P=0.032). Resistance to β-lactam antibiotics and β-lactam/β-lactamase combinations significantly correlated with biofilm formation capacity among enterococcal clinical isolates (P=0.039) (Table S3). However, there was no significant correlation between biofilm formation ability and resistance/sensitivity to other classes of antimicrobial agents.

### Clonal relationship analysis by RAPD-PCR

Enterococcal isolates recovered from different clinical sources were genotyped by the RAPD-PCR approach. Great diversity was observed in RAPD-PCR profiles of both *E. faecalis* (Figure 1) and *E. faecium* (Figure 2) isolates recovered from different clinical sources at an 85% cutoff. Some *E. faecalis* isolates showed a similarity coefficient higher than 85% and were considered clonal (for example: *E. faecalis* and *E. faecium*), however, their antibiotic susceptibility profiles and resistance determinants detected were different (Figure 1). No clustering of the enterococcal isolates was observed based on the clinical source from which they were recovered or their biofilm formation capacity.

### Discussion

Enterococci colonize the gastrointestinal tract of both humans and animals and are considered a part of its normal beneficial flora; however, it has emerged as a nosocomial pathogen in the last few decades. *E. faecalis* is the most commonly reported species accounting for more than 80% of enterococcal infections; however, *E. faecium* has also been increasingly reported which could be attributed to its resistance to multiple classes of antibiotics. In our study, *E. faecalis* was the most prevalent species detected, accounting for 68.9%, while 31.1% were identified as *E. faecium*. Most of the enterococcal isolates (54.4%) were collected from urine, followed by blood (35%), and wound (10.7%). Previous reports have also indicated that UTI is the most common infection caused by enterococci, followed by surgical site infections (SSI), bacteremia, and endocarditis.42-44

Recent reports have indicated that HLGR among enterococcal isolates ranged from 50% to 65%, while HLSR was less common than HLGR, ranging from 29% to 38%. Similarly, we had observed HLGR to be more predominant than HLSR. However, our study has indicated a higher rate of HLGR (79.6%) among enterococcal isolates in Egypt. Although enterococci have intrinsic resistance to low levels of aminoglycosides, high-level resistance is mediated by acquisition of aminoglycoside-modifying enzymes (AMEs) encoding genes. HLGR (MIC≥500 μg/mL) in enterococci is mostly mediated by the *aac(6')-Ie-aph(2'')-Ia* gene that encodes the bifunctional aminoglycoside modifying enzyme AAC(6')-APH(2'') and confers high-level resistance to aminoglycosides except streptomycin, while HLSR in enterococci are mediated by *ant(6')-Ia* gene. In enterococci, AMEs eliminate the synergistic activity of aminoglycosides when combined with a cell wall active agent, such as ampicillin or vancomycin.43,45,46

Increased resistance to macrolides, lincosamides, and streptogramin B (MLS₈ resistance) among enterococcal clinical isolates may be attributed to the wide use of this class of antimicrobials for treatment of enterococcal infections, except UTIs. MLS₈ resistance is attributed to active efflux, target modification, or inactivating enzymes. Methylation of 23S rRNA, mediated by *erm* gene, mediates resistance to macrolides, lincosamides, and streptogramin B. *ermB* is the most commonly reported gene among enterococcal isolates, while *ermA* and *ermC* are occasionally reported. *msrA/B* gene encodes efflux pump for macrolides and streptogramin B. Similar to previous reports, *ermB* and *msrA/B* genes were present in most macrolide resistant enterococcal isolates tested.45,47

Tetracyclines are broad-spectrum antibiotics and one of the most frequently used antimicrobials in Europe and in veterinary medicine. Tetracycline resistance determinants (*tet* genes) mediate ribosomal protection, enzymatic inactivation, and efflux of tetracyclines. *tetK* and *tetL* are involved in the efflux mechanism, while *tetM* plays a role in ribosomal protection among enterococci.50 Our data have indicated that most enterococcal clinical isolates (89.3%) carried more than one *tet* gene conferring different mechanisms of resistance to tetracyclines (efflux and ribosomal protection). There is
not enough data or reports describing the prevalence of tetracycline resistance and its determinants among enterococcal clinical isolates. Recently, Zahid et al.\(^5\) indicated that 57% of enterococcal isolates carried tetL, 50% carried tetM, while 33% carried both tetL and tetM.

Association of antibiotic resistance genes with mobile genetic elements increases the risk of dissemination of antibiotics resistance among enterococcal isolates. Tn916 was the first conjugative transposon, carrying the tetM gene, identified in \(E.\) \(faecalis\).\(^5\) Since then, a large number of transposable elements have been identified carrying resistance to different classes of antibiotics.\(^4\) In the present study, the integrase (\textit{int}) and the excisase (\textit{xis}) genes of Tn916 were detected in 72 isolates (69.9%) of enterococcal isolates collected from different clinical sources. A recent study has indicated that Tn916 was detected in 43.3% of enterococcal clinical isolates.\(^5\)

Enterococcal resistance to glycopeptides is mediated by \textit{van} operons.\(^3\) Most vancomycin resistant enterococcal outbreaks are attributed to \textit{vanA} and \textit{vanB} gene clusters.\(^4,5\) \textit{vanA} is inducible and confers high-level resistance to both vancomycin and teicoplanin, while \textit{vanB} confers a variable level of resistance to vancomycin, but not to teicoplanin.\(^3\) \textit{vanC} is less virulent than \textit{vanA} and \textit{vanB} and intrinsically present in some enterococcal species. However, the \textit{vanC} gene cluster has been recently detected in \(E.\) \(faecalis\) and \(E.\) \(faecium\) clinical isolates providing moderate level resistance to vancomycin.\(^5,6\) Variable rates of vancomycin resistance were detected across the world, with lower rates detected in Europe and Canada (4%–6%).\(^4,5,7\) A recent study in Iran has indicated that 18.8% of their enterococcal clinical isolates were resistant to vancomycin, where all isolates had MIC\(>\)256\(\mu\)g/mL and 90% of VRE carried the \textit{vanA} gene cluster.\(^4\) Our data showed high prevalence of VRE in Egypt (15.5%); however, VRE carried either \textit{vanB} or \textit{vanC} gene clusters. Moreover, our results coincide with previous reports that showed a higher rate of vancomycin resistance in \(E.\) \(faecium\) than \(E.\) \(faecalis\) clinical isolates.\(^4\)

Linezolid, the first oxazolidinone used clinically, is one of the most promising agents against MDR G+ve pathogens, especially VRE. However, linezolid resistance has been increasingly reported among enterococcal clinical isolates.\(^5,7,8\) Klare et al.\(^9\) found that the rate of linezolid resistance increased in Germany from \(<\)1% in 2008 to \(>\)9% in 2014. Our results coincide with their results, where 9.7% of our isolates were linezolid resistant. Mutation in domain V of the 23S rRNA gene, and transferable plasmid mediated methytransferase (\textit{cfr}) and \textit{optrA} genes have been reported to mediate resistance to oxazolidinones.\(^5,7\) Beside oxazolidinones, the \textit{optrA} gene also confers resistance to phenolics. It was first described in 2015 in enterococci recovered from human and animal origins in China.\(^7\) Afterwards, the \textit{optrA} gene has been detected in linezolid resistant enterococcal isolates in several places worldwide.\(^6\) In our study, the \textit{optrA} gene was detected in 80% of the linezolid resistant enterococci. To our knowledge, this is the first report of the \textit{optrA} gene among linezolid resistant enterococci in Egypt. The \textit{cfr} gene has been previously detected among linezolid resistant enterococci;\(^5,6,9\) however, it was not detected among linezolid resistant enterococci tested.

Enterococci have a high biofilm formation capacity, which increases the difficulty of management of enterococcal infections and hinders the penetration of antibiotics.\(^4,6\) In the present study, most of the enterococcal clinical isolates were biofilm producers, where 60.2% were strong/moderate biofilm producers. Several previous studies have indicated a high biofilm formation capacity among enterococcal clinical isolates.\(^6,7\) All linezolid-resistant strains exhibited strong/moderate biofilm formation. Moreover, vancomycin-resistant isolates were more likely to exhibit strong/moderate biofilm formation than vancomycin-sensitive isolates. A previous study has also indicated the correlation between biofilm formation capacity among enterococcal clinical isolates and resistance to linezolid.\(^6\) Another study has indicated that resistance to penicillin G, ampicillin, vancomycin, ciprofloxacin, chloramphenicol, and nitrofurantoin was significantly higher among biofilm producers than biofilm non-producers.\(^6\)

In this study, a higher level of resistance was observed among \(E.\) \(faecium\) than \(E.\) \(faecalis\) isolates (Table 2) as previously reported.\(^2,4,5,5\) Magiorakos et al.\(^10\) have defined MDR as non-susceptibility to at least one agent in three or more antimicrobial classes, while XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial classes. According to this definition, 53 \(E.\) \(faecalis\) isolates (74.6%) were MDR, while all \(E.\) \(faecium\) (N=32) were MDR strains. Two \(E.\) \(faecalis\) isolates (2.8%) and four \(E.\) \(faecium\) (12.5%) were resistant to all tested antimicrobials tested and considered as XDR (Table S4). Horizontal gene transfer is held responsible for the elevated levels of resistance to different antimicrobials, since plasmids and transposable elements usually carry resistance determinants for more than one class of antimicrobial agents. The increased level of resistance to vancomycin and linezolid highlights the problem of limited options to treatment infections caused by MDR and XDR enterococcal strains.
Conclusion
Antimicrobial susceptibility testing is of utmost importance in formulating an effective antibiotic policy for treatment of enterococcal infections and reducing morbidity and mortality. Neglecting standard protocols, empirical use of antibiotics, and lack of sufficient information are the leading causes for the emergence and spread of MDR and XDR enterococci in hospitals. Hence, effective measures, standard protocols, and programs to control the spread of enterococci should be implemented.

Disclosure
The authors report no conflicts of interest in this work.

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## Supplementary materials

### Table S1 Prevalence of antibiotic susceptibility among enterococcal species isolated from different clinical sources

| Antimicrobial agent | E. faecalis (N=71) | E. faecium (N=32) | P-value |
|---------------------|--------------------|-------------------|---------|
|                     | R (%) | I (%) | S (%) | R (%) | I (%) | S (%) |          |
| Ampicillin          | 21 (29.6) | 0 (0) | 50 (70.4) | 32 (100) | 0 (0) | 0 (0) | The chi-square statistic is 41.0091. The P-value is <0.00001. |
| Ampicillin/sulbactam | 21 (29.6) | 0 (0) | 50 (70.4) | 31 (96.9) | 1 (3.1) | 0 (0) | The chi-square statistic is 40.0396. The P-value is <0.00001. |
| Amoxicillin         | 19 (26.8) | 2 (2.8) | 50 (70.4) | 30 (93.8) | 2 (6.3) | 0 (0) | The chi-square statistic is 41.158. The P-value is <0.00001. |
| Amoxicillin/clavulanic acid | 19 (26.8) | 2 (2.8) | 50 (70.4) | 30 (93.8) | 2 (6.3) | 0 (0) | The chi-square statistic is 41.158. The P-value is <0.00001. |
| Imipenem            | 15 (21.1) | 0 (0) | 56 (78.9) | 30 (93.8) | 2 (6.3) | 0 (0) | — |
| Meropenem           | 19 (26.8) | 3 (4.2) | 54 (76.1) | 31 (96.9) | 1 (3.1) | 0 (0) | — |
| HLGR                | 57 (80.3) | 0 (0) | 14 (19.7) | 25 (78.1) | 0 (0) | 7 (21.9) | The chi-square statistic is 0.3891. The P-value is 0.823203. |
| HLSR                | 17 (24) | 0 (0) | 54 (76.1) | 21 (65.6) | 0 (0) | 11 (34.4) | The chi-square statistic is 16.6831. The P-value is 0.000238. |
| Erythromycin        | 66 (93) | 5 (7) | 0 (0) | 28 (87.5) | 4 (12.5) | 0 (0) | The chi-square statistic is 1.1451. The P-value is 0.564093. |
| Clarithromycin<sup>a</sup> | 40 (88.9) | 0 (0) | 5 (11.1) | 21 (80.8) | 0 (0) | 5 (19.2) | The chi-square statistic is 1.0435. The P-value is 0.593476. |
| Azithromycin<sup>a</sup> | 35 (77.8) | 1 (2.2) | 9 (20) | 18 (69.2) | 0 (0) | 8 (30.8) | The chi-square statistic is 1.0791. The P-value is 0.583011. |
| Clindamycin         | 71 (100) | 0 (0) | 0 (0) | 32 (100) | 0 (0) | 0 (0) | The chi-square statistic is 0.6366. The P-value is 0.722781. |
| Ciprofloxacin       | 60 (84.5) | 4 (5.6) | 7 (9.9) | 24 (75) | 0 (0) | 8 (25) | The chi-square statistic is 3.9361. The P-value is 0.139728. |
| Levofloxacin        | 63 (88.7) | 1 (1.4) | 7 (9.9) | 29 (90.6) | 1 (3.1) | 2 (6.3) | The chi-square statistic is 0.714478. |
| Tertracycline<sup>a</sup> | 66 (93) | 4 (5.6) | 1 (1.4) | 30 (93.8) | 2 (6.3) | 0 (0) | The chi-square statistic is 0.722781. |
| Doxycycline<sup>a</sup> | 33 (46.5) | 25 (35.2) | 13 (18.3) | 24 (75) | 7 (21.9) | 1 (3.1) | The chi-square statistic is 8.2472. The P-value is 0.016186. |
| Vancomycin          | 1 (1.4) | 4 (5.6) | 66 (93) | 6 (18.8) | 5 (15.6) | 21 (65.6) | The chi-square statistic is 14.2318. The P-value is 0.000812. |
| Linezolid           | 4 (5.6) | 0 (0) | 67 (94.4) | 6 (18.8) | 0 (0) | 26 (81.3) | The chi-square statistic is 4.628. The P-value is 0.098863. |

Notes:  R: Resistant; I: Intermediate resistance; S: Sensitive.  
<sup>a</sup>E. faecalis vs E. faecium; P-value < 0.05 was considered statistically significant.  
<sup>b</sup> Susceptibility for clarithromycin and azithromycin was performed on 71 isolates only.

Abbreviations:  HLGR, High Level Gentamicin Resistance; HLSR, High Level Streptomycin Resistance.

### Table S2 Prevalence of biofilm formation among enterococcal isolates collected from different clinical sources

| No. (%) of enterococcal isolates with biofilm formation capacity | E. faecalis (N=71) | E. faecium (N=32) | P-value |
|---------------------------------------------------------------|--------------------|-------------------|---------|
|                                                               | Strong | Moderate | Weak | Biofilm producers | Biofilm non-producers |          |
| E. faecalis (N=71)                                            | 13 (18.3) | 29 (40.9) | 24 (33.8) | 66 (93) | 5 (7) | The chi-square statistic is 1.1294. The P-value is 0.769975. |
| E. faecium (N=32)                                             | 8 (25) | 12 (37.5) | 11 (34.4) | 31 (96.9) | 1 (3.1) |          |
| Total (N=103)                                                 | 21 (20.4) | 41 (39.8) | 35 (34) | 97 (94.2) | 6 (5.8) |          |

Note:  E. faecalis vs E. faecium; P-value < 0.05 was considered statistically significant.
Table S3 Correlation between biofilm formation capacity and antibiotic susceptibility among enterococcal clinical isolates

| Term          | AM R (53) | SAM R (53) | AX R (53) | AMC R (53) | IPM R (47) | MEM R (54) | VA R (16) | HLGR R (82) | HLSR R (38) | S_BP S (50) | M_BP S (50) | W_BP S (50) | N_BP S (50) | chi² P  |
|---------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-------------|-------------|-------------|-------------|-----------|---------|
| S_BP          | 15        | 6         | 15        | 6         | 15        | 6         | 15        | 6         | 15        | 6           | 15          | 6           | 15          | 6         | 8.353   |
| M_BP          | 17        | 24        | 17        | 24        | 17        | 24        | 17        | 24        | 17        | 24          | 17          | 24          | 17          | 24        | 8.353   |
| W_BP          | 20        | 15        | 20        | 15        | 20        | 15        | 20        | 15        | 20        | 15          | 20          | 15          | 20          | 15        | 8.353   |
| N_BP          | 1         | 5         | 1         | 5         | 1         | 5         | 1         | 5         | 1         | 5            | 0           | 6           | 0           | 3         | 8.353   |

Note: P-value < 0.05 was considered statistically significant.

Abbreviations: AM, Ampicillin; AMC, Amoxicillin/clavulanic acid; AX, Amoxicillin; AZM, Azithromycin; CIP, Ciprofloxacin; CLR, Clarithromycin; DA, Clindamycin; DO, Doxycycline; E, Erythromycin; HLGR, High Level Gentamicin resistance; HLSR, High Level Streptomycin resistance; IPM, Imipenem; LEV, Levofloxacin; LZD, Linezolid; M_BP, Moderate biofilm producer; MEM, Meropenem; N_BP, Non-biofilm producer; R, Resistant; S, Sensitive; S_BP, Strong biofilm producer; SAM, Ampicillin/sulbactam; TE, Tetracycline; VA, Vancomycin; W_BP, Weak biofilm producer.

Table S4 Distribution of multidrug resistance (MDR) and extensive drug resistance (XDR) among enterococcal clinical isolates

| Term          | E. faecalis (n=71) | E. faecium (n=32) |
|---------------|---------------------|-------------------|
| Resistance profile | No. (%) of isolates | Resistance profile | No. (%) of isolates |
| g, s, a, c, F, T, V, l | 2 (2.8) | g, s, a, c, F, T, V, l | 4 (12.5) |
| g, s, a, c, F, T, l | 1 (1.4) | g, a, c, F, T, V, l | 1 (3.1) |
| g, s, a, c, F, T | 4 (5.6) | g, s, a, F, T, V | 4 (12.5) |
| g, a, c, F, T | 9 (12.7) | g, a, T, V, l | 1 (3.1) |
| g, s, a, F | 1 (1.4) | g, a, F, T | 5 (15.6) |
| a, c, F, T | 1 (1.4) | F, T, l | 1 (3.1) |
| g, F, T | 18 (25.4) | F, T | 8 (11.3) |
| g, s, F | 2 (2.8) | F, V | 1 (1.4) |
| c, F, T | 1 (1.4) | g | 2 (2.8) |
| a, F, T | 1 (1.4) | — | 1 (1.4) |

Abbreviations: A, Penicillins (ampicillin); C, Carbapenems (Imipenem or Meropenem); F, Fluoroquinolones (Ciprofloxacin or Levofloxacin); G, High Level Gentamicin resistance; L, Oxazolidinones (Linezolid); S, High Level Streptomycin resistance; T, Tetracyclines (Doxycycline); V, glycopeptides (Vancomycin).
Figure S1 Agarose gel electrophoresis for detection of antimicrobials resistance determinants among enterococcal isolates. (A). Detection of antimicrobials resistance encoding genes among enterococcal isolates by PCR. Lane M is Molecular weight marker. 1: aac(6')-Ie-aph(2'')-Ia (200 bp); 2: ant(6')-Ia (597 bp); 3: tetK (370 bp); 4: tetL (715 bp); 5: tetM (170 bp); 6: ermB (639 bp) and 7: msrA/B (400 bp); 8: optrA (1395 bp); 9: ins-Tn (1046 bp), and 10: ins-Tn (194 bp) genes that are used for detection antimicrobials resistance among enterococcal isolates. (B). Multiplex PCR for detection of vancomycin resistance determinants (vanA (1030 bp), vanB (536 bp), vanC1 (822 bp), and vanC2/3 (484 bp)), E. faecium-specific (658 bp), E. faecalis-specific (941 bp) and of rrs (320 bp) genes. Lane M is Molecular weight marker. 1: E. faecalis isolate; 2: E. faecium isolate; 3: E. faecium vanB isolate; 4: E. faecalis vanC1 isolate; 5: E. faecium vanC1 isolate; 6: E. faecalis vanB isolate.