

RESEARCH ARTICLE

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The Antimicrobial Resistance and Prevalence of *Enterococcus* Species in Saudi Arabia

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Abstract

Monitoring the distribution and resistance of antibiotics to enterococcal species is critical aspect to controlling and preventing enterococcal infection. The aim of the present study is to screen the antimicrobial resistance genes within *Enterococcus* species isolates that collected from Taif governorate, Saudi Arabia. Out of 134 clinical samples, nineteen enterococcal isolates were identified using 16S rRNA sequence gene. Phylogenetic tree analysis using 16S rRNA gene sequence of the 19 strains divided them into 15 strains as *E. faecalis* and 4 strains as *E. faecium*. In addition, these the species of these isolates were recognized using VITEK-2 COMPACT system. The PCR technique was used to screen the multi-drug resistant genes within enterococcal isolates. The *KpsII*, *tetL*, *aac(6)-Ie-aph(2)-Ia*, *vanA* and *Erm(B)* genes were found in all strains. The distribute of resistance against antibiotic drugs were differs greatly between the two species, a considerably higher prevalence of resistance to penicillin, gentamicin, cefoxitin, cefotaxime, clindamycin, erthromycin and fusidic acid was identified in *E. faecalis* than in *E. faecium*, while greater spread was detected to resist to Trim/Sulf and tetracycline in *E. faecalis*. Finally, rep-PCR markers investigated genomic diversity of *Enterococcus* strains. Results of rep-PCR markers generated 142 distinct loci; 96 were polymorphic (67.6%) and 46 were monomorphic (32.4%). Number of loci for individual rep-PCR primers ranged from 9 for rep-08 to 18 for rep-02.

Keywords: *Enterococcus* spp.; antimicrobial resistance; Antibiotic resistance genes, Saudi Arabia.

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INTRODUCTION

The genus *Enterococcus* consists of thirty-eight species. The most common identified living microorganisms usually found in the intestines of human and animal cells including *E. faecalis* and *E. faecium*^{1,2}. *E. faecalis* is accountable for about 80% of human infections². Intestinal microorganisms are opportunistic organisms that become pathogenic due to an imbalance in the immune system. Currently, Olawale et al.³ reported that intestinal tract infections can cause infections in hospitals, especially in urinary tract, surgical sites and bloodstream. Several reports pointed out that gastroenteritis is the third organism isolated between infection in hospitals of Saudi Arabia and the most isolated microorganisms common in the bloodstream^{4,5}. Approximately 90% of intestinal endocarditis occurs due to *E. faecalis*, with less than 5% affected by *E. faecium*⁴. Infrequently, additional species are documented in disease conditions, including *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae*, *E. mundtii* and *E. malodoratus*⁶. Primarily in the last twenty years, these strains caused infections in the hospitals of Saudi Arabia. *Enterococcus* comprises a necessary piece of the gastrointestinal microflora of people, animals and insects. Notwithstanding their reality as commensals in the gastrointestinal tract, a few strains exist as sharp pathogens. In a previous study, vancomycin resistance to intestinal microorganisms (VRE) accounts for about 43% of all intestinal catheter isolates, an amount that rises due to unavailability of vancomycin for clinical use in USA. Two types of VRE isolates are included; *E. faecium* and *E. faecalis*. Both species were isolated to resist eight antibiotics³. Antibiotic resistance may be help in Enterococci development and consider as a threat to public health. The high distribution of *Enterococcus* spp. has developed as a public health threat⁷. *Enterococcus* is the most common bacterial infection in hospitals⁷. *Enterococcus* types have many virulence factors such as gastrointestinal protein and aggregation content, which increases the colonization process of epithelial lining of host cells⁸. The gene coding for ESP was commonly identified in medical infection isolates⁹. Antibiotic resistance factors are transferred by mergers, plasmids or transposons that can act as carriers. These genes are transferred to other members of

the same microbial species and transmission of horizontal genes may arise through conjugation, transduction and transformation^{5,10}.

The polymerase chain response (PCR) is a less complex method that gives results inside a short timeframe and at lower costs. By this technique, diverse primer sits, homologous, aleatory or degenerated, are utilized to intensify locales of the DNA particle. Band designs are in this way delivered, allowing the gathering of comparative strains and the separation of irrelevant ones¹¹. Repetitive (REP) sequences are extragenic units found in various locales of the DNA of bacterial species¹². The amplification of the areas between these units creates a helpful unique fingerprint to separate *Enterococcus* strains¹³.

The purpose of the present research was to screen the distribution of the antimicrobial resistance genes within *Enterococcus* spp in patients from King Faisal Hospital in Taif governorate, Saudi Arabia. Moreover, the genetic diversity between the *Enterococcus* spp. strains were investigated using rep-PCR markers.

MATERIAL AND METHOD

Molecular identification of *Enterococcus* strains

Out of 134 bacterial isolates, nineteen multiple-drug resistant enterococcal strains were obtained from King Faisal Hospital in Taif governorate, Saudi Arabia. Isolates were recognized by high-throughput using a VITEK-MS (bioMérieux, France) system according to manufacturer's protocol. The DNA extraction kit (Gena Bioscience, Germany) was used to isolate genomic DNA from all 19 isolates accordance the manufacturer's directions. For each isolate and as per previously described methods, one fragment of 16S rRNA gene (about 1465 bp) was amplified from each stain as previously reported¹⁴. The specific band was purified from the gel using QIAGEN purification kit (QIAGEN, USA). The purified band was sequenced via DNA Analyzer 3146 (Applied Biosystems, USA). To perform the BLAST searched, NCBI service was used (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). The phylogenetic tree was constructed using neighbor joining algorithm with Kimura 2 parameter distances in MEGA 7.1 software. The bar indicates the Juke-Cantor evolutionary distance.

Antibiotic Susceptibility Testing

All nineteen *Enterococcus* strains were screened for antimicrobial susceptibility using an automated VITEK-2 (bioMérieux) system. In this protocol, broth microdilution minimum inhibitory concentration (MIC) method for susceptibility testing and accomplish repetitive turbidimetric screen of bacterial growth through an abbreviated incubation period. MIC results were taken based on the Clinical and Laboratory Standards Institute guidelines¹⁵. The criteria of Hassan et al. were used to defined MDR isolates¹⁶. The susceptibility of *Enterococcus* isolates to 16 antibiotics including vancomycin, gentamycin, cefoxitin, efotaxime, ampicillin, daptomycin, trime/sulf, teicoplanin, vancomycin, clindamycin, erythromycin, fusidic acid, linezolid, nitrofurantoin, ciprofloxacin, moxifloxacin and tetracycline was determined.

Detection of Antimicrobial Resistance Genes

Five virulence genes [*KpsII*, *tetL*, *aac(6)-Ie-aph(2)-Ia*, *vanA*, and *Erm(B)*] were detected in all *Enterococcus* strains in multi-Drug resistant according to Hassan et al.¹⁴. The resistance genes against antibiotic were detected in all 19 strains as previously reported⁴. The antibiotic resistance genes were isolated from the 19 strains by PCR with primers synthesized by the macrogen Co., Ltd. (Seoul, Korea) (Table 1). The PCR reaction for each gene was accomplished as previously described⁴.

Rep-PCR analysis

The settings of rep-PCR technique for the 19 strains of *Enterococcus* were standardized. Five primers (Rep-1, Rep-2, Rep-8, Rep-12 and Rep-18) were used to investigate the genetic variability between the *Enterococcus* strains. The primers sequence and PCR conditions were performed as previously reported¹⁷.

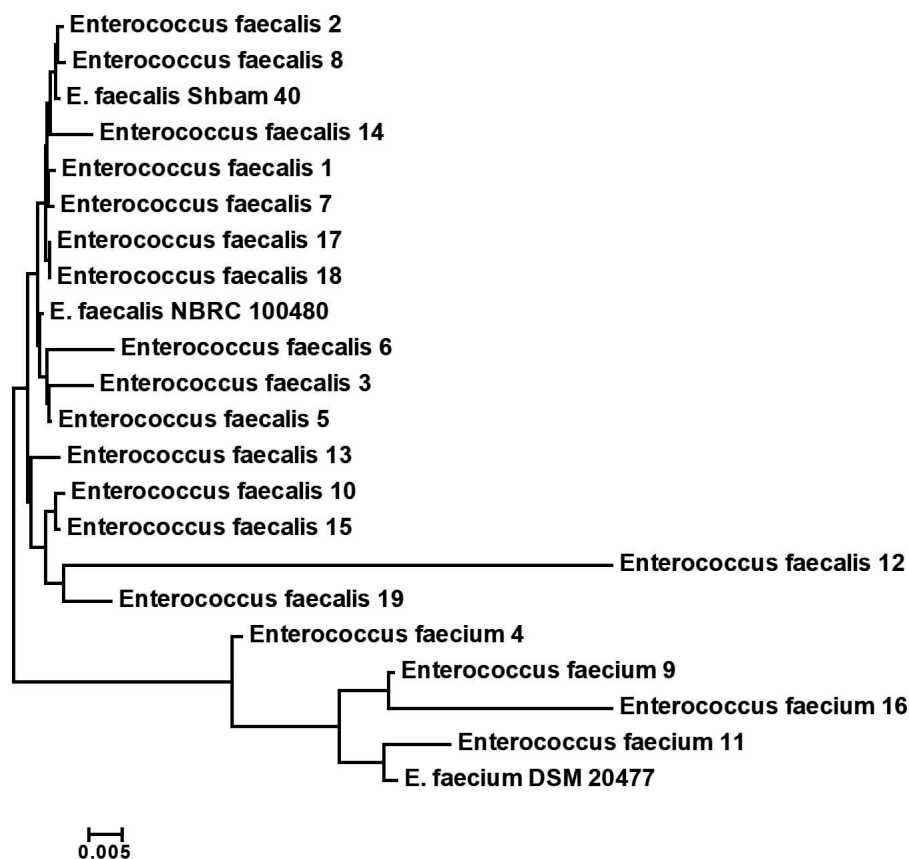


Fig. 1. Phylogenetic relationship of the multi-Drug resistance *Enterococcus* isolates and related genera based on full size 16S rDNA sequences. The tree was constructed using neighbor joining algorithm with Kimura 2 parameter distances in MEGA 7.1 software. The bar indicates the Juke-Cantor evolutionary distance.

RESULTS

Enterococcus isolates identification

The species of the nineteen *Enterococcus* isolates were known by a fully automated VITEK-2 COMPACT microbiology method. Fifteen were recognized as *E. faecalis* and four as *E. faecium*. These results were confirmed by 16S rRNA sequencing. The 16S rRNA gene sequence of all 19 isolates was compared with other *Enterococcus* strains that placed in GenBank database. The results were somehow differed from the obtained sequence (data not shown). Ribosomal genes are greatly relevant for investigating bacterial evolution and phylogeny. When re-constructing phylogenetic relationships of microorganisms, sequencing of 16S rRNA has been generally

employed. Phylogenetic tree analysis using 16S rRNA sequences of all 19 strains and other related *Enterococcus* species from GenBank were positioned the present *Enterococcus* strains into *E. faecalis* and *E. faecium* (Fig. 1).

Antibiotics sensitivity test

All *Enterococcus* strains showed resistance to the majority of selected antibiotics (Fig. 2). A significantly higher resistance to gentamicin, cefoxitin, cefotaxime, clindamycin, erythromycin and fusidic acid were found in *E. faecalis* than in *E. faecium*. On the contrary, a higher resistance to trim/sulf and tetracycline was observed in *E. faecium*. Furthermore, a low resistance to nitrofurantoin, linezolid and daptomycin was noticed in both species. In addition, these isolates

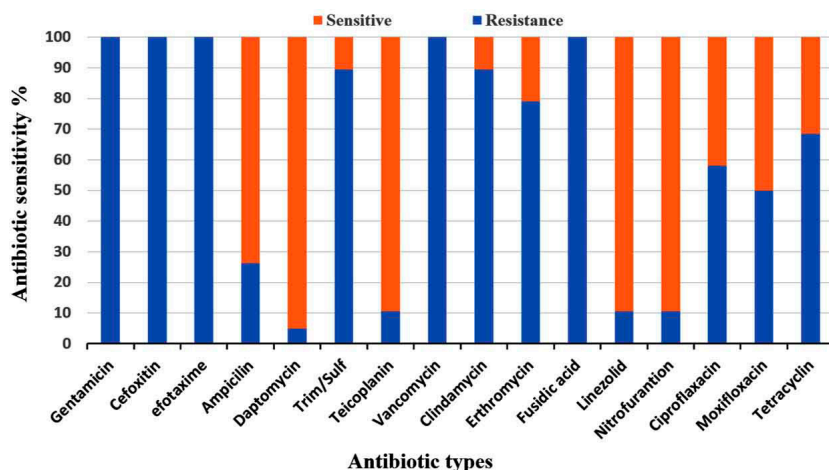


Fig. 2. Antimicrobial resistance profiles of nineteen multi-Drug resistance *Enterococcus* strains against sixteen antibiotics.

Table 1. PCR primers for detection of some antibiotic resistance and virulence genes in *Enterococcus* spp. isolates

Genes	Primer sequence (52'→32')	Size (bp)
<i>Tuf</i>	TACTGACAAACCATTTCATGATGAACCTTCGTCACCAACGCGAAC	112
<i>KpsII</i>	GCGCATTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272
<i>Dd1-E</i>	CAAAGTGTGGCATTCCACAA TGGATTTCCTTTCCAGTC ACTTC	550
<i>Dd2-E</i>	GAAGAGCTGCTGCAAAATGCTTTAGCGCGCGCTTCAATTCCTTGT	941
<i>Van-A</i>	GTAGGCTGCGATATTCAAAGC CGATTCAATTGCGTAGTCCAA	231
<i>Erm(B)</i>	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	405
<i>aac(6)-Ie-aph(2)-Ia</i>	CAGAGCCTTGGGAAGATGAAG CCTCGTGAATTCATGTTCTGGC	348
<i>tet(L)</i>	GTMGTTGCGCGCTATATCC GTGAAMGRWAGCCACCTAA	696

Notes: M = A or C; R = A or G; W = A or T *Tuf* gene specific for *Enterococcus*; *KpsII* gene specific for capsule formation in *Enterococcus* species; *Dd1-E* gene specific for *Enterococcus faecalis*; *Dd2-E* gene specific for *Enterococcus faecium*; *Van-A* gene specific for vancomycin resistance; *erm(B)* gene specific for erythromycin resistance; *aac(6)-Ie-aph(2)-Ia* gene specific for gentamycin resistance; and *tet(L)* gene specific for tetracycline.

were found to be less than 50% sensitivity to ciprofloxacin, moxifloxacin and tetracycline (Fig. 2 and 3).

Antimicrobial resistance genes prevalence

The *KpsII*, *tetL*, *ermB*, *aac(60)-le-aph(200)-la* and *vanA* genes were recognized in all isolates. The identification of these genes in both species were showed in table 2. The *ermB* gene was found in the majority of ciprofloxacin-resistant enterococci, indicating the occurrence of different systems related to the resistance of enterococci to fluoroquinolones. Similar gene were present in 79% among the erythromycin-sensitive enterococci and 10.5% among the

linezolid-sensitive enterococci, demonstrating no expression of *ermB* gene in some *enterococcus* strains. Further, *ermB* gene occurred at a greater and significance role in fluoroquinolone-resistant enterococci compare to that in the sensitive species. Therefore, the existence of *ermB* gene was connected with the protection against fluoroquinolones in the *Enterococcus* species (Fig. 4).

Rep-PCR analysis

Rep-PCR markers were used to investigate the genomic diversity of *Enterococcus* strains. Table 3 and Figure 4 illustrates rep-PCR results. Rep-PCR markers produced 142 distinct loci; 96 bands were polymorphic (67.6%) and merely 46 bands were monomorphic (32.4%). Individual rep-PCR primers included a number of bands varying from 9 bands for rep-08 to 18 bands for rep-02. The greatest polymorphism marker was recorded for rep-08, rep-12 and rep-18. The band size of rep-18 ranged between 350 to 2100 bp (Fig. 5). The lowest polymorphism (62.5%) was detected in rep-01. Primers rep-1, rep-8, rep-12 and rep-18 produced equal mono morphism and polymorphism bands. The band size of rep-12 ranged between 100 to

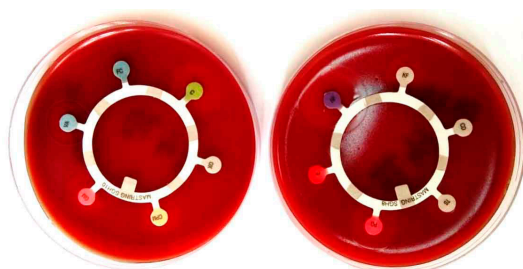


Fig. 3. The combination disk diffusion test of *Enterococcus* strain with some antibiotics

Table 2. Antimicrobial resistance profiles of the 19 *Enterococcus* isolates

Isolates	Antibiotic resistance genes							
	<i>Tuf</i>	<i>KpsII</i>	<i>Dd1-E</i>	<i>Dd2-E</i>	<i>Van-A</i>	<i>Erm(B)</i>	<i>aac(6)-le-aph(2)-la</i>	<i>Tet(L)</i>
<i>Enterococcus faecalis</i> (E1)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E2)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E3)	+	+	+	-	+	+	+	+
<i>Enterococcus faecium</i> (E4)	+	+	+	+	+	+	+	+
<i>Enterococcus faecalis</i> (E5)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E6)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E7)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E8)	+	+	+	-	+	+	+	+
<i>Enterococcus faecium</i> (E9)	+	+	-	+	+	+	+	+
<i>Enterococcus faecalis</i> (E10)	+	+	+	-	+	+	+	+
<i>Enterococcus faecium</i> (E11)	+	+	+	+	+	+	+	+
<i>Enterococcus faecalis</i> (E12)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E13)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E14)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E15)	+	+	+	-	+	+	+	+
<i>Enterococcus faecium</i> (E16)	+	+	-	+	+	+	+	+
<i>Enterococcus faecalis</i> (E17)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E18)	+	+	+	-	+	+	+	+
<i>Enterococcus faecalis</i> (E19)	+	+	+	-	+	+	+	+

1400 bp, while, the band size of rep-18 ranged between 350 to 1700 bp (Fig. 5).

The phylogenetic analysis tree using Neighbor Joint method according to the bands that produced from rep-PCR markers was shown in Fig. 6. A low genetic distance, ranged from 0.02 to

0.36, among *Enterococcus* isolates was determined based on Jaccard's similarity coefficients (Fig. 6). The *Enterococcus* strains were gathered into two groups with around 66% genetic similarity. The first group was included *E. faecium* samples (Entero-4, 9, 11 and 16), with similarity 76%. While, the

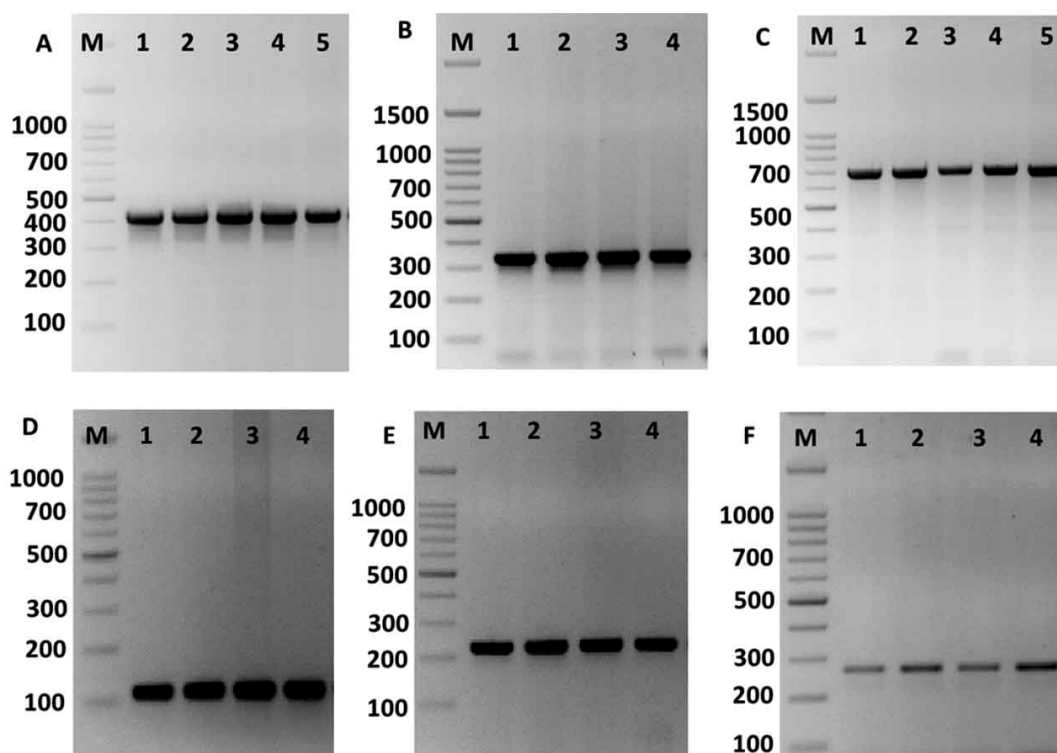


Fig. 4. Amplification of some specific genes producing in some *Enterococcus* isolates by single PCR. (A) *ErmB* gene specific for erythromycin resistance with size about of 405 bp. (B) *aac(60)-le-aph(200)-la* gene specific for gentamicin with size about of 348 bp. (C) *TetL* gene specific for tetracycline resistance with size about of 696 bp. (D) *Tuf* gene specific for *Enterococcus* spp. with size about of 112 bp. (E) *VanA* gene specific for vancomycin resistance with size about of 231 bp. And (F) *KpsII* gene specific for capsule formation in *Enterococcus* species with size about of 272 bp. First lane on each panel is 100 bp molecular weight markers

Table 3. Polymorphic bands of each genetic primers and percentage of polymorphism in nineteen *Enterococcus* spp. isolates based on the five rep-PCR primers

Primers	Total Bands	No. of Monomorphic Bands	No. Polymorphic Bands	% Monomorphic Bands	% Polymorphic Bands
Rep-1	16	6	14	37.5	62.5
Rep-2	18	10	8	35.7	64.3
Rep-8	9	6	3	33.3	66.7
rep-12	16	6	10	33.3	66.7
rep-18	14	6	8	33.3	66.7
Total	142	46	96	32.4	67.6

second main group included *E. faecalis* samples, that divided into two clusters. The first cluster contained Entero-14, 15, 17, 18 and 19 isolates, and the second one contained Entero-1, 2, 3, 5, 6, 7, 8, 10, 12 and 13. These results suggested that mostly genetic distance between native *Enterococcus* strains was quite low among each species, while, was high between the two species (Fig. 6).

DISCUSSION

Out of 134 clinical isolates, 19 antiretroviral strains, 15 as *E. faecalis* and 4 as *E. faecium*, were obtained from King Faisal Hospital in Taif, Saudi Arabia, within four months. The antimicrobial resistance of *Enterococcus* spp. was recognized to be common in gastrointestinal tracts^{2,14,18}. The frequency of both species in 206 strains of intestinal tract infections that obtained

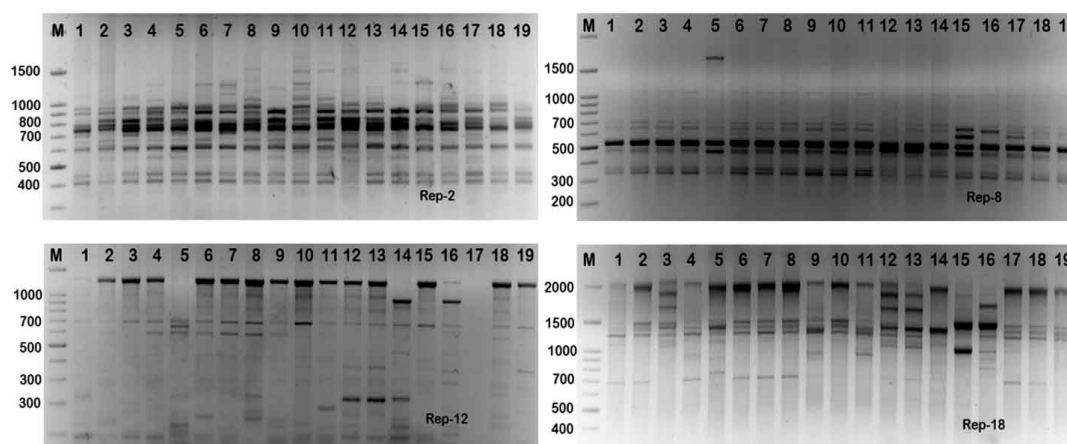


Fig. 5. Rep-PCR profile of 19 *Enterococcus* spp. isolates generated with four rep primers, rep-1, rep-2, rep-8, rep-12 and rep-18, respectively. First lane on each panel is 100 bp molecular weight markers.

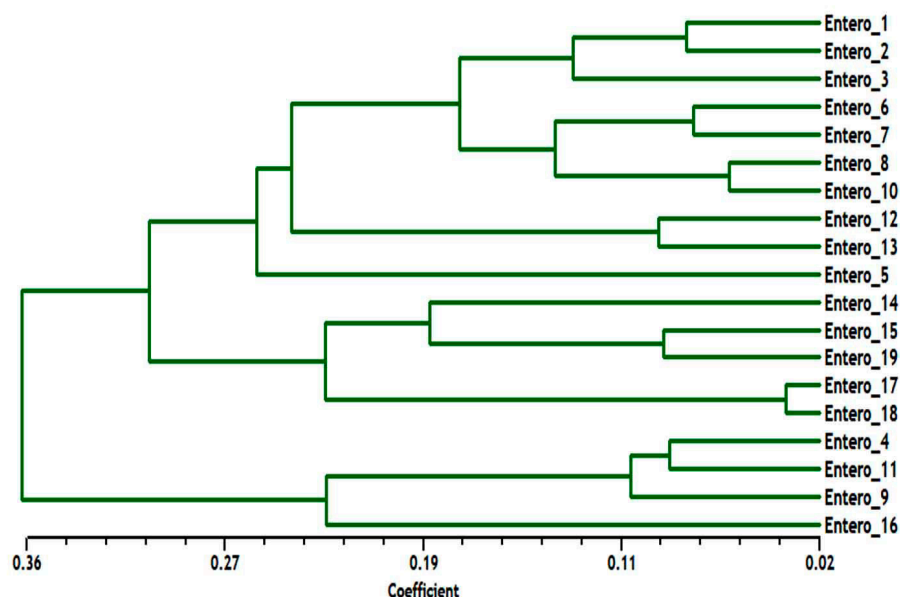


Fig. 6. Dendrogram analysis among nineteen *Enterococcus* spp. isolates generated with five rep-PCR primers, *E. faecium* samples included (Entero-4, 9, 11 and 16), *E. faecalis* samples included (Entero-1, 2, 3, 5, 6, 7, 8, 10, 12, 13, 14, 15, 17, 18 and 19).

from clinical specimens at Riyadh hospitals, KSA, were 69.2% for *E. faecalis* and 11.3% for *E. faecium*¹⁹. Also, the different microorganism that isolated from intestinal patients of the University Hospital in Taif, Saudi Arabia were documented as *E. faecium* with 34% and *E. faecalis* with 33%⁴. In agreement to these reports, the main two species, *E. faecium* and *E. faecalis*, in our clinical specimens were found to be a natural resistance to gentamicin and aminoglycosides. Although bacteria found sensitive to these drugs in laboratory trials, effectiveness was initiate to be unsatisfactory in clinical practice^{20,21}. *Enterococcus* species has been reported as widely multiple-antimicrobial resistance^{4,22}. In the present study, a greater level of resistance to gentamicin, cefoxetin, ceftotaxim, clindamycin, erythromycin and fucidic acid was detected in *E. faecalis* than *E. faecium*, while a greater resistance occurrence towards Trim/Sulf was detected in *E. faecium*. The resistance of *E. faecalis* to tetracycline was greater than *E. faecium*. Furthermore, a low predominance of protection against linezolid and nitrofurantoin was identified in both species. In this way, linezolid, vancomycin, and nitrofurantoin are as of now generally utilized for viable treatment of intestinal fistula infection^{23,24}. To achieve an understanding of the change in antimicrobial resistance in gastrointestinal tracts in different hospitals, a comparison of our study with other clinical intestinal strains that have resistance towards antimicrobial agents and collected from January 2014 to December 2015 was made⁴. There was a significant increase percentage of *Enterococcus* strains, where *E. faecium* was remain prevalent, but the component rate enlarged. Persistent antibiotic pressure is believed to lead to secondary resistance to gastrointestinal microbes^{4,25}. Here, *ermB* gene was found in all intestinal cysteine resistance to ciprofloxacin. Moreover, the existence of the *ermB* gene was highly significant in strain of enterococci that showed resistance to fluoroquinolone than those sensitive strains. Currently, many aminoglycoside adjusting enzymes have been recognized that have tow functions of 6'-aminoglycoside acetyltransferase (AAC(6')) and 2"-aminoglycoside phosphotransferase (APH(2'')). These enzymes usually encoded by *aac(6'')/aph(2'')* gene. This gene typically removes the synergistic consequence between glycopeptide or penicillin antibiotics

and aminoglycosides⁵. The incidence of *aac(60)-le-aph(200)-la* gene was detected in all strains. Here, the occurrence of tetracycline-resistant genes remained in *E. faecium* with 10.5% and in *E. faecalis* with 89.5%. Consequently, protection of intestinal tract species against tetracyclines is believed to be as a result of the existence of a gene that encodes the relating enzymes. One of the mechanisms that responsible for the resistance of macrolides in intestinal microorganisms is the alteration of erythromycin target site by *ermB* and *mef* genes in the genome of enterococci microorganisms²⁶. In this study, *vanA* gene was identified in all 19 strains. Vancomycin-resistant *Enterococcus* species have been able to transfer *vanA* gene to *Staphylococcus aureus*, leading to the appearance of vancomycin-resistant *S. aureus* bacteria, leading to further difficulty in the clinical treatment of intestinal infections^{4,26}. As a result, care should be taken when vancomycin is used as a drug for treatment of gastrointestinal infection and management of viomycin-resistant enterococci need to be enhanced²³.

Molecular procedures used to identify bacterial strains vary as far as unfair power, reproducibility, standardization, cost, simplicity of advancement and interpretation²⁷. REP-PCR were established and demonstrated great outcomes when utilized to type *Enterococcus* strains¹³. There are a few other PCR-based strategies effectively applied for enterococcal separation, for example RAPD-PCR²⁸, ITS-PCR²⁹, tDNA-PCR³⁰, AFLP³¹, and PCR-RFLP³². Additionally, multilocus arrangement examination (MLSA) is likewise a procedure utilized for recognizable proof of enterococci by utilizing partial sequences of RNA polymerase alpha subunit, phenylalanyl-tRNA synthase and the alpha subunit of ATP synthase^{33,34}. In any case, PCR results are progressively hard to examine, since the presence of different powerless loci in the PCR profiles makes it hard to decipher the results. Rep-PCR with oligonucleotide primers used for amplified genomic DNA from enterococci and demonstrated acceptable results when used to identify *E. faecium* clinical isolates³⁵. Here, the Rep-PCR method approved to be a satisfied technique to discriminate *Enterococcus* spp., however, additional studies should be assumed to evaluate strains obtained from other hospitals.

CONCLUSION

Enterococci have been the major pathogenic microorganisms that make clinical contaminations because of various antimicrobial resistance, and the clinical enterococcal diseases prevalently happen in the urinary framework. Antimicrobial sensitivity changes in various *Enterococcus* species, and the resistance of enterococci to antimicrobial antibiotics is essentially inferable from the development of antimicrobial resistance genes. The screening of antimicrobial resistance of *Enterococcus* species would give a manual for the fitting determination of antimicrobial agents and avert the existence of increasingly antimicrobial-resistant enterococcal strains. The present results propose a moderately high predominance of antibiotic resistance in enterococci strains, especially to gentamicin, vancomycin, cefoxitin, and efotaxime. It is of worry that the scope of antibiotics to which resistance has been gained after some time is generally wide, to the degree that it presently incorporates new developing antibiotics utilized for the treatment of enterococcal contamination.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

None.

AUTHOR'S CONTRIBUTION

WFA and EMF designed the study. AS, MA, HH and KA contributed to the collected and analysis of the clinical samples. MAF performed the molecular analysis. WFA and EMF contributed in the literature research and aided in draft the manuscript. WFA, MA and HH contributed to data analysis and manuscript with the final version. All authors read and accepted the final manuscript.

DATA AVAILABILITY

All datasets obtained or studied during this study are incorporated in the manuscript.

ETHICS STATEMENT

This study was accepted by the ethics council of obstetrics hospital in King Faisal complex, Taif, Saudi Arabia.

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