Electronic Journal of Biotechnology 18 (2015) 175–180

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Electronic Journal of Biotechnology

Clonal diversity and antimicrobial resistance of *Enterococcus faecalis* isolated from endodontic infections

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ARTICLE INFO

Article history:
Received 13 November 2014
Accepted 14 January 2015
Available online 31 March 2015

Keywords:
Antimicrobial susceptibility
Endodontic infection
Enterococcus faecalis
Genotype
RAPD
RFLP

ABSTRACT

Background: Enterococcus faecalis is considered to be one of most prevalent species in the oral cavity, particularly in endodontic infections. The aim of the present study was to investigate the prevalence of *E. faecalis* in dental root canals, clonal diversity by restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD-PCR) analysis, and the antibiotic susceptibility of *E. faecalis* isolates.
Results: Among the bacterial strains isolated from dental root canal specimens (n = 82), *E. faecalis* was determined to have the highest prevalence followed by *Streptococcus viridians*, *Leuconostoc mesenteroides*, *Staphylococcus aureus*, *Streptococcus mitis*, and *Pediococcus pentosaceus*. Cluster analysis of RAPD-PCR and RFLP patterns of the *E. faecalis* isolates discriminated five and six different genotypes, respectively. Among the tested strains, 43%, 52% and 5% were susceptible, intermediate resistant, and resistant to erythromycin, respectively. In addition, one strain (E-12) was intermediate resistant to linezolid, and one isolate (E-16) was resistant to tetracycline. Interestingly, many of the intermediate resistant/resistant strains were grouped in clusters 5 and 6, according RAPD and to RFLP, respectively.
Conclusions: E. faecalis demonstrated the highest prevalence in the tested dental root canal specimens collected from Saudi patients and were grouped into five to six different genotypes. Different levels of antimicrobial susceptibility were observed in the tested *E. faecalis* strains, which clearly indicated that although bacterial strains may be similar, point mutations can result in extreme susceptibility or resistance to various antibiotics. This phenomenon is a cause for concern for clinicians in the treatment of dental infections caused by *E. faecalis*.

1. Introduction

Enterococci are Gram-positive, coccus-shaped bacteria and are common colonizers of different animal hosts, plants, soil, food, and water [1,2]. Previously, these bacteria were considered as normal commensals of the gastrointestinal tract, oral cavity, and vagina in humans [3,4]. However, there was recently an increase in nosocomial infections caused by enterococcal species, including urinary tract, bloodstream, endocardium, abdomen, biliary tract, burn wound, and endodontic infections, which were largely attributed to the antimicrobial resistance profiles [5,6,7]. Therefore, Enterococci now rank among the top three nosocomial bacterial human pathogens, and several multidrug-resistant strains have emerged that pose great therapeutic challenges [8,9]. Among various enterococcal species, *Enterococcus faecalis* is considered the most prevalent species due to its pathogenicity and high frequency of isolation from infection sites; up to 90% of enterococcal infections in humans are caused by *E. faecalis* [10,11]. One of those important infection sites is the oral cavity, particularly in endodontic infections [12,13,14].

E. faecalis is a non-fastidious, therapy-resistant microorganism in infected root canals. It has been reported as the species that is most commonly recovered from teeth with failed endodontic treatment and persistent infections, particularly in individuals with periodontal diseases [4,9,15]. However, data obtained for the oral prevalence of *E. faecalis* vary widely in different studies. In addition, the status of the oral cavity influences, directly or indirectly, the colonization by *E. faecalis*. For instance, enterococci were detected in samples from multiple oral sites in 60% of school children with high caries activity and in 75% of patients with endodontic infection [16].

Genomic fingerprints are increasingly used to study relationships at the intra- or even the inter-specific level. Differences in these fingerprints between individuals are interpreted as genetic distances [17]. However, the applied methods should provide the appropriate level of discriminatory power and be relatively efficient and cost

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.03.004
0717-3458/

effective. Several methods are available for DNA finger printing, including restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD-PCR), which have been described as powerful molecular typing methods for microorganisms and have become the "gold standard" for molecular typing [18,19,20]. Unfortunately, there are insufficient data concerning the prevalence of *E. faecalis* in Saudi patients with endodontic infection. Therefore, the aim of the present study was to investigate the prevalence in endodontic infections in Saudi patients, the clonal diversity by RFLP and RAPD-PCR analysis, and the antibiotic susceptibility of the isolated *E. faecalis* strains.

2. Materials and methods

2.1. Patient selection

In the present study, clinical dental root canal samples were collected from patients who were admitted to the College of Dentistry Clinics, King Saud University (Riyadh, Saudi Arabia). Written consent was obtained from each patient, and the study was approved by the 'Ethical Committee of the College of Dentistry Research Center'. The clinical dental specimens were collected during the period from June 2011 to May 2012. Eighty-two samples were collected from root canals, including 56 necrotic, 21 retreated, and five vital teeth. None of the patients had received antibiotic therapy during the preceding three months. The patients consisted of 67 males and 15 females and ranged in age from 16 to 72 years.

2.2. Clinical and sampling procedures

Endodontic samples were obtained from root canals according to a previously reported approach [14,21,22]. A K-type file (#15) with the handle cut off was initially used for the sample collection. A sterile file (#15) was used to agitate the canal contents for 30–60 s. The file was introduced to a level about 1 mm short of the tooth apex based on diagnostic radiographs, and a gentle filing motion was applied. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal to ensure viable sample acquisition. Next, two sequential paper points were placed at the same level and retained in position for 60 s to soak up the fluid in the canal. All of the collected clinical samples were immediately transferred to sterile 2-mL Eppendorf tubes containing VMGA III transport medium. The VMGA III transport medium contained gelatin (5.0 g), thione E peptone (0.05 g), thioglycolic acid (0.05 g), L-cysteine-HCl (0.05 g), Na-glycerol-phosphate (1.0 g), phenylmercuric acetate (0.0005 g), methylene blue (0.0003 g), $CaCl_2$ $6H_2O$ (0.024 g), KCl (0.042 g), NaCl (0.1 g), $MgSO_4$ $7H_2O$ (0.01 g), and agar (0.2 g).

2.3. Bacterial isolation and identification

The Eppendorf tubes containing the clinical dental samples in VMGA III transport medium were initially pre-incubated for 30 min at 37°C and shaken vigorously in super mixer for 60 s. Next, 10-fold serial dilutions were made in 1% sterile peptone water (Bacto peptone, Difco, USA), and 0.1 mL of each dilution was distributed over the blood agar plates. The plates were incubated in an anaerobic jar (Oxoid, England) at 37°C for 72 h. The obtained colonies were sub-cultured several times on fresh blood agar plates until homogeneous colonies were obtained. The purity of the cultures was confirmed by Gram staining, colony morphology, and hemolytic activity on blood agar. Glycerol cultures of all of the isolates were prepared and stored at -80°C for further analysis. All of the isolated clinical bacterial strains were subjected to identification using biochemical tests and a Vitek® 2-C15 automated system for bacterial identification (BioMerieux Inc., France), according to manufacturer's instructions [11,17].

2.4. Antimicrobial susceptibility testing

Susceptibility of the isolated clinical *E. faecalis* strains (n = 21) toward various antimicrobial agents (n = 9) was investigated using the agar disc diffusion method and Vitek® 2-C15 automated system, including the following antibiotics: levofloxacin (Lev), moxifloxacin (Mox), erythromycin (Ery), linezolid (Lzd), teicoplanin (TI), vancomycin (VA), tetracycline (Tet), tigecycline (Tig), and nitrofurantoin (NI) [23,24]. The bacterial strains were sub-cultured on fresh Mueller–Hinton agar plates (Difco, USA) for 24 h at 37°C. After the incubation period, the cells were collected using a sterile loop and suspended in sterile saline solution to be equivalent to 0.5 McFarland standards. The cell suspensions were inoculated onto Mueller–Hinton agar plates using sterile cotton swabs, and various antibiotic discs were placed (in duplicate) carefully on the agar plate surfaces and incubated for 24 to 48 h at 37°C. *Staphylococcus aureus* was used as a control microorganism. The minimum inhibitory concentrations (MICs) of the various antibiotics (n = 9) against the isolated *E. faecalis* strains were determined using automated Vitek 2-C15 configuration (BioMerieux Inc., France). Breakpoint concentrations of various antibiotics and MIC values indicating susceptibility and resistance were evaluated according to Clinical and Laboratory Standard Institute (CLSI) guidelines [25].

2.5. Genotyping of the isolated E. faecalis strains

The *E. faecalis* isolates were grown overnight at 37°C in brain heart infusion broth (Oxoid LTD, Hampshire, England). The cells were

Table 1
Prevalence and distribution of various bacterial strains isolated from root canals, including necrotic, retreated, and five vital teeth.

Isolated clinical strains	No. of isolated strains according to source			Total No. of isolates	Prevalence (%)
	Necrotic (n = 56)	Retreated (n = 21)	Vital (n = 5)		
Enterococcus faecalis	19	2 (strains E-3 and E-5)	0	21	25.6
Gemella morbillorum	1	0	0	1	1.2
Kocuria kristinae	1	1	0	2	2.4
Leuconostoc spp.	0	1	0	1	1.2
Leuconostoc mesenteroides	8	5	0	13	15.9
Pediococcus pentosaceus	3	4	0	7	8.5
Staphylococcus aureus	3	3	5	11	13.4
Staphylococcus haemolyticus	0	0	1	1	1.2
Streptococcus mitis	2	4	2	8	9.8
Staphylococcus sciuri	0	0	1	1	1.2
Streptococcus viridans	8	4	4	16	19.5
Total	45	24	13	82	100.0

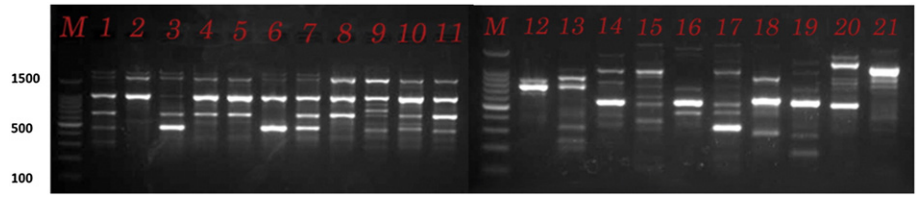

Fig. 1. Random amplification of polymorphic DNA (RAPD-PCR) patterns of the isolated clinical *E. faecalis* (n = 21). M: 100 bp ladder.

harvested (maximum of 2×10^9 cells) in micro-centrifuge tubes by centrifugation for 10 min at 10,000 rpm, and the supernatant was discarded. The bacterial pellets were washed twice with sterile distilled water, and the total DNA of all of the strains was extracted and purified using DNeasy Blood & Tissue Kits (QIAGEN, USA) according to the manufacturer's instruction. The purity and efficacy of the total extracted DNA were tested using agarose gel electrophoresis. Genotyping of the isolated *E. faecalis* strains was conducted by RAPD-PCR and RFLP analysis.

2.5.1. RAPD-PCR analysis

The *E. faecalis* isolates were typed by RAPD-PCR using a RAPD specific primer, 5′-GGTGACGCAG-3′, according to a previously reported method [19,26]. The purified total DNA from *E. faecalis* strains (n = 21) was used as a template in the RAPD-PCR reaction. The RAPD-PCR reactions were performed in 25 μL volumes containing 12.5 μL GoTaq® Green Master Mix (Promega, USA); 2 μL DNA (50 μg/mL), 2 μL RAPD primer (BioLabs, England), and 8.5 μL nuclease-free water (Promega, USA). The amplification conditions consisted of an initial denaturation step at 94°C for 120 s, followed by 50 cycles of 95°C for 1 min (denaturation), 36°C for 30 s (annealing), and 72°C for 1 min (elongation); followed by a final elongation step for 10 min at 72°C. The RAPD-PCR products were analyzed by 2% agarose gel electrophoresis.

2.5.2. RFLP analysis

RFLP analysis of the isolated *E. faecalis* strains (n = 21) was performed in two steps including (i) amplification and purification of the 16S rDNA genes of all of the strains, followed by (ii) digestion of the purified 16S rDNA amplicons with ApaI restriction enzyme and analysis of the digests using agarose gel electrophoresis [17].

2.5.2.1. PCR amplification of 16S rDNA gene. The 16S rDNA genes from the isolated *E. faecalis* strains (n = 21) were PCR-amplified using the universal eubacterial forward primer 16F27 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 16R1525 (5′-AAG GAG GTG ATC CAG CCG CA-3′) derived from the *E. coli* 16S rDNA sequence synthesized by MWG Biotech AG (Germany). The PCR amplification was performed using the purified genomic DNA of the *E. faecalis* strains as templates. The PCR reaction (25 μL) contained 12 μL of PCR master mix (Promega, USA), 2 μL of forward primer, 2 μL of reverse primer, 2 μL of DNA templates, and 6.5 μL of nuclease-free water. The PCR reaction was performed in a thermal cycler (MWGAG Biotech, USA) using the following conditions: initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s; annealing at 52°C for 30 s; extension at 70°C for 1.5 min, and then, a final extension step at 70°C for 5 min. The PCR products (12 μL) were mixed with 3 μL of loading dye solution (Qiagen, USA) and analyzed by 1% (w/v) agarose gel electrophoresis using a 1 kbp DNA ladder (Qiagen, USA) as molecular size standard. The gel was placed in ethidium bromide solution (1 μg/mL) for 30 min, and then on an ultraviolet transilluminator to visualize the DNA. The amplified 16S rDNA products were removed from the agarose with a sterile razor blade, and the DNA was purified from the agarose using QIAquick gel Extraction Kits (Qiagen, USA) according to the manufacturer's instructions [17,27].

2.5.2.2. Digestion of purified 16S rDNA with ApaI restriction enzyme. The purified amplified 16S rDNA of the *E. faecalis* isolates (n = 21) was digested with ApaI restriction enzyme (Promega, USA) according to the manufacturer's instructions. Briefly, 12.8 μL of nuclease-free water was added to sterile PCR tubes, followed by the addition of 2 μL RE 10× buffer (Promega, USA), 0.2 μL acetylated BSA (Promega, USA), 3 μL DNA, and 2 μL of ApaI (Promega, USA). The mixtures were mixed by pipetting, centrifuged for a few seconds in a micro-centrifuge and incubated in a water bath at 37°C for 4 h. The ApaI digestion products of the 16S rDNA amplicons were analyzed using 2% agarose gel electrophoresis, and the gels were stained with ethidium bromide and photographed [17,27].

2.5.3. Cluster analysis

Differences between isolates were determined by visual inspection of the bands obtained from RAPD-PCR and RFLP and with the PyElph

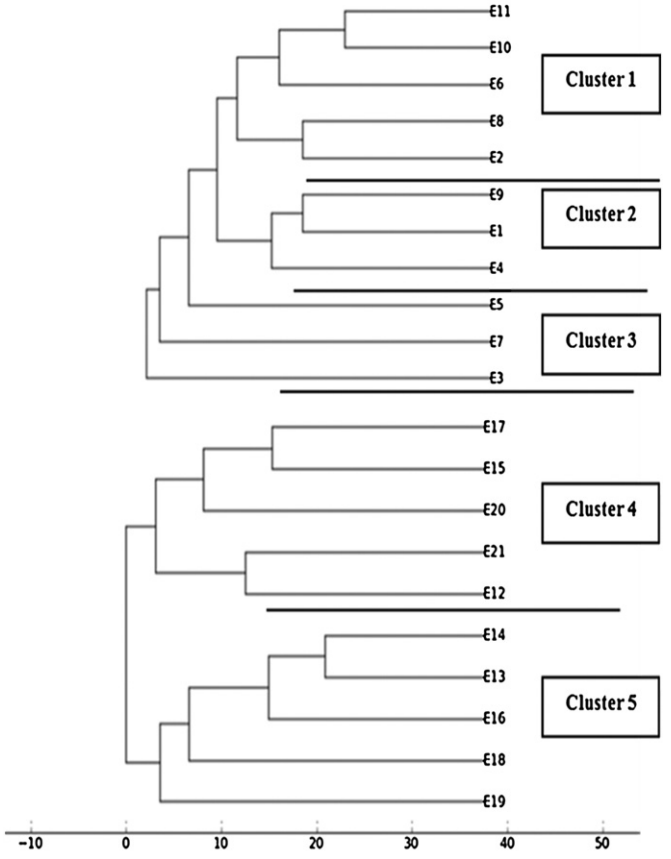

Fig. 2. Dendrogram of the *E. faecalis* isolates (n = 21) from RAPD-PCR patterns using the Pearson product moment correlation coefficient (r) and the UPGMA.

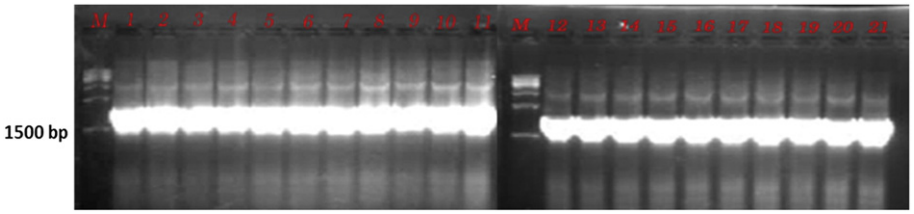

Fig. 3. Gel electrophoresis of the amplified 16S rDNA of the isolated *E. faecalis* isolates (n = 21). Lane M: 1 kbp.

software tool as recommended by Van Belkum et al. [28,29]. Calculation of the similarity of the band profile and grouping of the RAPD-PCR and RFLP patterns was based on the Dice correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis [28].

3. Results and discussion

3.1. Microbial isolation and identification

Eighty-two clinical dental samples were collected from root canals, including 56 specimens from necrotic, 21 retreated, and five vital teeth. The enrichment and bacterial isolation from the collected specimens resulted in the isolation of 82 non-repetitive bacterial isolates. Among the isolated bacterial strains, *E. faecalis* was found to have the highest frequency and distribution (n = 21; 25.6%), followed by *Streptococcus viridians* (n = 16; 19.5%), *Leuconostoc mesenteroides* (n = 15; 9%), *S. aureus* (n = 11; 3.4%), *Streptococcus mitis* (n = 9; 9.8%), *Pediococcus pentosaceus* (n = 7; 8.5%), and other bacteria representing less than 3% of the total bacterial numbers (Table 1). The majority of the *E. faecalis* strains were found in necrotic root canals (n = 19), from which two strains were isolated from retreated root canals (strains E-3 and E-5); no *E. faecalis* strain were found in specimens collected from the vital teeth (Table 1). The prevalence of the various microorganism detected in the root canals in the present study was relatively similar to that reported by Ercan et al., who found that *E. faecalis* had the highest frequency followed by *Streptococcus* sp. and *Staphylococcus* sp. [15]. In addition, the prevalence and distribution of *E. faecalis* (25.6%) in root canals in our study were similar to that reported by Zoletti et al., who detected *E. faecalis* in 25% of the tested dental root canals [8]. In addition, Cogulu et al. identified *E. faecalis* in 26% of the tested necrotic teeth using the culture method [12]. Furthermore, our results were in agreement with other findings reported elsewhere [23,30,31]. However, in a recent study, *E. faecalis* was detected at very low numbers in untreated canals [16]. A comparison between the distribution frequency of the clinical isolates of *E. faecalis* (n = 21) in male and female patients indicated that this bacterium was detected at much higher frequency in male compared to female patients. Among 15 root canal samples collected from females, two *E. faecalis* strains were isolated, while 19 strains were isolated from 67 males.

3.2. RAPD-PCR and RFLP genotyping of the isolated E. faecalis strains

Genetic variations in microorganisms lead to several phenomena that are clinically very significant and demanding [17,32]. In the present study, genomic DNA from the isolated *E. faecalis* strains (n = 21), including 19 strains from necrotic tissues and two strains from retreated root canals (strains E-3 and E-5), was extracted, and the purity of the DNA was confirmed using agarose gel electrophoresis. The purified bacterial DNA was used as the template for the analysis of *E. faecalis* clonal diversity by RAPD-PCR and RFLP. The diversity of the RAPD-PCR products generated by the selected RAPD primer for the isolated clinical *E. faecalis* strains was high, with patterns showing between five and seven well-separated bands (Fig. 1). The cluster analysis of the RAPD-PCR patterns of the 21 *E. faecalis* strains discriminated five different genotypes (Fig. 2). However, restriction RFLP of the isolated *E. faecalis* strains (n = 21) was conducted in two steps, including the amplification and purification of the 16S rDNA genes, followed by the digestion of purified 16S rDNA amplicons with ApaI restriction enzyme and analysis of the digests by agarose gel electrophoresis. As shown in Fig. 3, 16S rDNA genes of all of the isolated clinical *E. faecalis* strains (n = 21) were successfully amplified, showing the expected gene length of 1525 bp [17]. The analysis of ApaI digestion products of the amplified 16S rDNA genes provided a RFLP pattern of three to five DNA fragments (Fig. 4). Cluster analysis of the RFLP patterns of the *E. faecalis* strains discriminated the 21 strains into six different genotypes (Fig. 5). *E. faecalis* strains E-3 and E-5 isolated from retreated canals were detected in one genotype according to the RAPD pattern (cluster 3), in which they were distributed between clusters 1 and cluster 2 according, to the RFLP pattern analysis. Many fingerprinting methods have been applied to study the microbial biodiversity. Within this context, RAPD and RFLP have been shown to be reliable tools for microbial identification and typification [27,32]. However, grouping of the same strains in different clusters based on the employed techniques has also been previously reported [8,32].

3.3. Antimicrobial susceptibility pattern of the isolated clinical E. faecalis isolates

The results of the susceptibility of the isolated clinical E. faecalis strains (n = 21) toward various antimicrobial agents (n = 9) are shown in Fig. 6. Different levels of antimicrobial susceptibility were observed in the tested *E. faecalis* strains (n = 21) according to the MIC

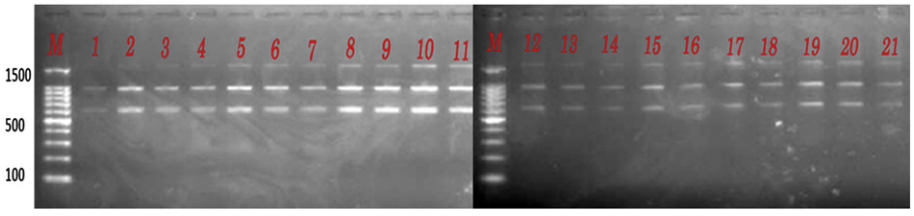

Fig. 4. Restriction patterns of the purified 16S rDNA genes of the E. faecalis isolates (n = 21). The purified amplified 16S rDNA of the isolated *E. faecalis* was digested with ApaI restriction enzyme, and the digestion products were analyzed by agarose gel electrophoresis. Lane M is ladder (100 bp).

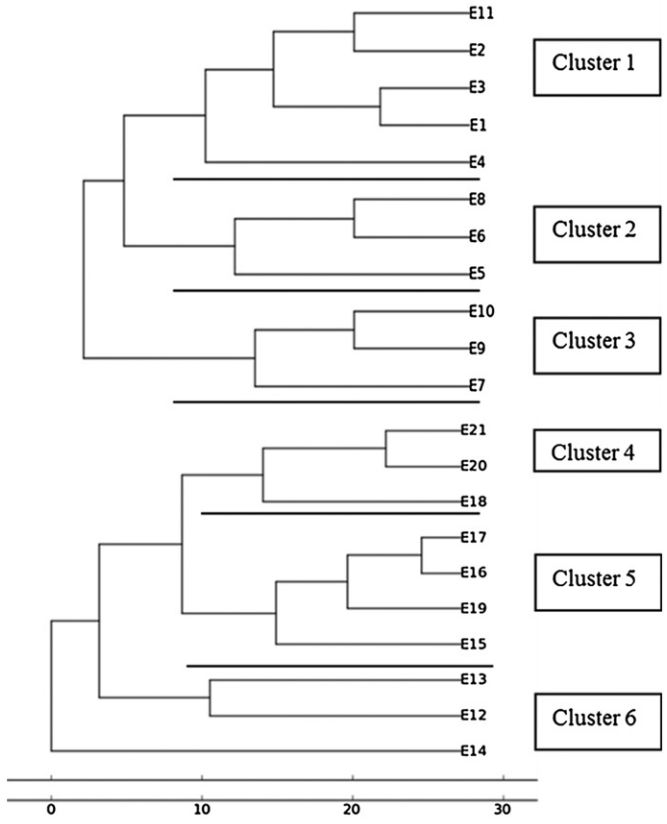

Fig. 5. Dendrogram of the *E. faecalis* isolates (n = 21) from RFLP patterns using the Pearson product moment correlation coefficient (r) and the UPGMA.

values, i.e., susceptible, intermediate resistant, and resistant. The results indicated that all of the *E. faecalis* isolates (n = 21) were susceptible to levofloxacin, moxifloxacin, teicoplanin, vancomycin, tigecycline, and nitrofurantoin. However, nine strains (43%) were susceptible, 11 strains (52%) were intermediate resistant (strains E-12, E-14, E-16, E-18, E-19, E-10, E-11, E-1, E-7, E-8, and E-9), and one strain (5%) (E-13) was resistant to erythromycin. In addition, among the tested *E. faecalis* isolates, one isolate (E-12) showed intermediate resistance to linezolid, and one isolate (E-16) was resistant to tetracycline. In the present study, there was a relatively high prevalence of isolates with intermediate resistance to commonly used antibiotics (erythromycin) in the treatment of oral *E. faecalis* strains, which is consistent with previous findings [23,33]. However, the emergence of a higher percentage of resistant *E. faecalis* strains has also been reported in previous studies [7,34]. In general, a significant increase in antimicrobial resistance is observed in the oral microflora, particularly in bacteria associated with periodontal diseases [35,36,37]. It is believed that the wide use of tetracyclines has led to the spread of resistance determinants, such as in the treatment of localized aggressive periodontitis and respiratory tract infections [38,39]. The incidence of resistance to tetracycline has been increasing annually. For example, between 2000 and 2010, several studies have reported that *E. faecalis* isolated from endodontic infections exhibit resistance to tetracycline at levels increasing from 14.3%, 15.1%, 28.8% and 30% [5,13,31]. Moreover, a recent study reported by Lins et al. showed that 70% of the isolated clinical *E. faecalis* strains were resistant to tetracycline, and 100% of the isolated strains were resistant to erythromycin [7].

Interestingly, many of the *E. faecalis* isolates with intermediate resistance/resistance to erythromycin and resistance to tetracycline were found to be among cluster 5 (according RAPD) and clusters 5 and 6, according to the RFLP patterns. These results clearly indicate that the morphology, physiology and biochemical reactions of bacterial strains may be similar, but a point mutation resulting in a change in the DNA base pair regimen can result in organisms that are either very susceptible or very resistant to many antibiotics, raising concerns for clinicians [40].

4. Conclusions

Bacterial isolation from dental root canal samples resulted in the identification of 82 non-repetitive clinical bacterial isolates. Among the isolated bacterial strains, *E. faecalis* displayed the highest frequency and distribution, followed by *S. viridians*, *L. mesenteroides*, *S. aureus*, *S. mitis*, and *P. pentosaceus*. The cluster analysis of the RAPD-PCR and RFLP patterns of the 21 *E. faecalis* isolated discriminated five and six different genotypes, respectively. Different levels of antimicrobial susceptibility were observed in various *E. faecalis* isolates. In addition, the emergence of strains with intermediate resistance/resistance to commonly used antibiotics against *E. faecalis* was observed. Many of the intermediate resistant/resistant strains were grouped in one genotype, which clearly indicated that a point mutation in the same strains can increase either

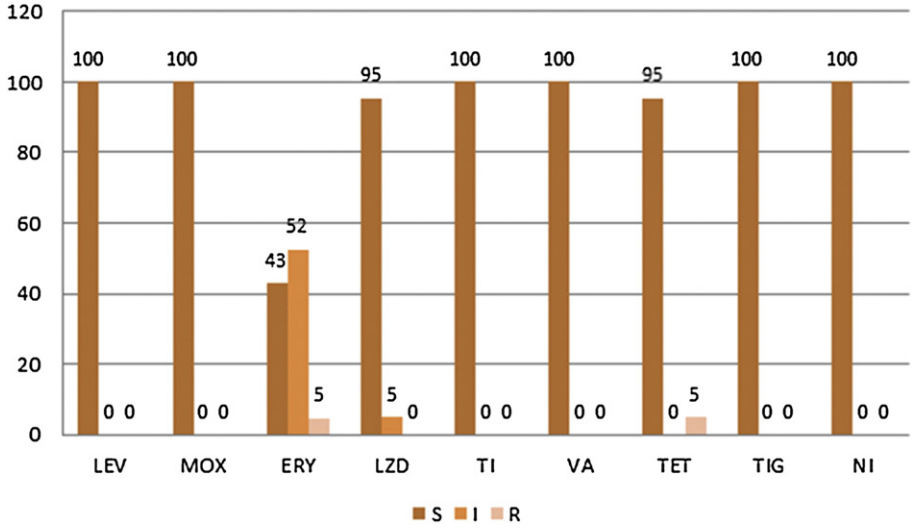

Fig. 6. Percentage of susceptible (S), intermediate (I) and resistant (R) strains among the isolated clinical *E. faecalis* isolates (n = 21). Susceptibility of the isolated clinical *E. faecalis* strains (n = 21) toward various antimicrobial agents (n = 9). LEV: levofloxacin; MOX: moxifloxacin; ERY: erythromycin; LZD: linezolid; TI: teicoplanin; VA: vancomycin; TET: tetracycline; TIG: tigecycline; and NI: nitrofurantoin.

susceptibility or resistance to various antibiotics, thus raising concerns for clinicians in the treatment of dental infections caused by *E. faecalis*. In addition, the known pathological potential of *E. faecalis* isolated from other body sites and the increasing multi-resistance to antibiotics highlights the need for microbiological diagnoses of root canal infections to identify *E. faecalis* at the clonal level and on the treatment of resistant bacteria, such as enterococci.

Financial support

The authors extend their appreciation to the Research Center at College of Science, Deanship of Scientific Research at King Saud University for funding this work.

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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