Comparison of genotypes, antimicrobial resistance and virulence profiles of oral and non oral *Enterococcus faecalis* from Brazil, Japan and the United Kingdom.

Short title:

Genotypic and phenotypic features of oral and non oral *Enterococcus faecalis*

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ABSTRACT

Objectives: To determine whether phenotypic and genotypic differences amongst isolates of *Enterococcus faecalis* relate to geographical and clinical origin. **Methods:** *E. faecalis* from primary endodontic infections in Brazilian patients (n=20), oral infections in UK patients (n=10), and non-oral infections in Japanese patients (n=9) were studied. In addition, 20 environmental vancomycin resistant *Enterococcus faecalis* (VRE) isolates from a UK hospital were analysed. For all isolates, polymerase chain reaction (PCR) was used to detect genes associated with antibiotic resistance and virulence, whilst randomly amplified polymorphic DNA-PCR (RAPD-PCR) was used to produce molecular profiles. **Results:** Gelatinase gene (*gelE*) was prevalent amongst isolates (77-100%) and for oral isolates, genes of aggregation substances (*agg*), immune evasion protein (*esp*), cytolysin (*cylB*), tetracycline resistance (*tetM*; *tetL*) and erythromycin resistance (*ermB*) were detected to varying extent. Japanese non-oral isolates had a similar genetic profile to oral isolates, but with higher prevalence of *ermB* and *cylB*. All VRE isolates were positive for *gelE, esp, agg, vanA, ermB* and *tetM*, 95% were positive for *cylB* and 17% positive for *tetL*. All isolates were negative for *ermA, asa373 vanB, vanC1 and vanC2/3*. RAPD-PCR revealed clustering of VRE isolates. **Conclusions:** RAPD-PCR analysis revealed extensive genetic variability among the tested isolates. Oral isolates carried antibiotic resistance genes for tetracycline and whilst they possessed genes that could contribute to pathogenicity, these were detected at lower incidence compared with non-oral and VRE isolates. RAPD-PCR proved to be a useful approach to elucidate relatedness of disparate isolates.

Keywords: *Enterococcus faecalis*; Genotype; Antimicrobial susceptibility; Endodontic infection.

INTRODUCTION

Enterococcus faecalis is an important nosocomial pathogen that has been implicated in oral infections, especially endodontic disease. However, little is known about the relatedness of *E. faecalis* isolates from different clinical origins, and the source and the role of these microorganisms in oral infection (1, 2, 3).

Enterococci are not considered normal inhabitants of the oral cavity but have been isolated from various oral conditions including carious lesions, periodontitis and root canal infection (4, 5). Although endodontic infections are usually mixed infections with a predominance of facultative and obligate anaerobic bacteria, it is the association of enterococci, particularly *Enterococcus faecalis* with both primary and secondary endodontic infection (6, 7) that has been most extensively documented.

There remains a paucity of knowledge about the role of enterococci in the pathogenesis of endodontic infections. Kayaoglu and Orstavik, 2004 (8), reviewed the virulence factors of *E. faecalis* that may be relevant in the context of endodontic infection and the periradicular inflammatory response. The most studied virulence factors are aggregation substance, surface adhesins, sex pheromones, lipoteichoic acid, production of extracellular superoxide, gelatinase and hyaluronidase, and the cytolysin toxin. Each of these factors may be associated with various stages of endodontic infection in addition to periapical inflammation. Some virulence factors are encoded at the *E. faecalis* pathogenicity island (PAI), from which large and horizontally transmitted elements can contribute to the rapid evolution of nonpathogenic organisms into pathogenic forms (9).

Understanding the source of the endodontic isolates is important to minimise the dissemination of virulent and multidrug resistant clones to the oral cavity. The prevalence of *E. faecalis* in the root canal system was shown to be associated with the

its presence in saliva (10), but an endogenous source has been questioned (11). Zehnder & Guggenheim, 2009 (12), proposed foodborne transmission, but evidence for this could not be provided in the study of Vidana et al 2016 (1) where a large number of oral and food isolates were profiled. In a separate study, the same research group concluded that whilst the potential for nosocomial transmission of enterococci from environmental surfaces in dental surgeries appeared small, further investigation of this was warranted (13).

Regardless of the source of endodontic *E. faecalis* we cannot exclude the possibility that pathogenic and multiple resistant strains may colonise the root canal environment, creating a potentially dangerous infection reservoir. In addition to a role in endodontic disease, subsequent systemic colonization and infection related to an oral source of enterococci is not unrealistic. Okui et al, 2015 (14) reported a case of infective endocarditis of oral origin caused by *E. faecalis.* Using RAPD-PCR, the authors confirmed that isolates from blood culture samples and from the gingival and buccal mucosa were identical.

RAPD analysis is a technique to rapidly detects genomic polymorphisms. Whilst many methods are available for molecular profiling of microorganisms, RAPD-PCR approaches are powerful, rapid, have low cost and are accessible. Furthermore they can be applied in many research environments and with minimal equipment. As such, RAPD-PCR methods have often been considered as ‘gold standards’ in molecular typing and analysis by this method is widely accepted and regarded as a reliable tool for differentiating and identifying enterococci (15). However, these approaches have not been extensively used for oral enterococci.

In this study, we hypothesised that oral strains can carry multiple virulence genes and exhibit close genetic relatedness to isolates from hospital environments and

hospitalised patients. The findings of the RAPD-PCR typing were analysed in the context of the presence of virulence factors to assess whether oral enterococcal infections could serve as reservoirs for the emergence and spread of virulent clones.

2. MATERIAL AND METHODS

2.1 Sample collection

A total of 59 *E. faecalis* isolates was studied. These included 20 endodontic isolates from patients with primary endodontic infection in Brazil (7), and 10 oral isolates from United Kingdom (including 4 isolates from saliva and 6 isolates from root canal and gifted from the School of Dentistry at King's College London, School of Dentistry, Cardiff University, and Glasgow Dental Hospital). From hospitalised patients in Japan, 9 non-oral isolates were also studied. This latter included *E. faecalis* isolated from urine, wound, genital and abdominal exudate (Kanazawa University Hospital). Twenty environmental VRE isolates were also included from the University Hospital of Wales (Cardiff. UK) (16).

2.2 Confirmation of *E. faecalis* identity by sequencing of 16S ribosomal DNA

Total DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Paisley, UK) and the manufacturer's recommended protocol. Extracted DNA served as template for PCR using the universal bacterial 16S rRNA primer pair of 27f and 1492r (Table 1). All PCRs were performed in a 50-µL final reaction volume containing 1 µM of each primer, 25 µL of PCR Master Mix (Promega, Madison, USA) and 2 µL of total DNA template. Negative controls of sterile ultrapure water instead of DNA template were included with each PCR. PCR was undertaken in a DNA thermocycler using an initial denaturation step of 95°C for 1 min, and then 26 thermal cycles of 94°C for 45s, 50°C for 45s and 72°C for 1.5 min, followed by a final step of 72°C for 15 min. Gel electrophoresis of the amplicons was performed in a 1.0% (w/v)

agarose gel at 60 V/cm^2. PCR amplicons stained with safe view dye (NBS biologicals, Cambridgeshire, UK) were subsequently detected by ultraviolet transillumination. A 100-bp DNA ladder (Promega) served as a molecular weight standard.

Enterococcus species were identified using Eurofins MWG Operon's sequencing service. Briefly, PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and the DNA subjected to sequencing PCR using the primer 357f (Table 1). The partial 16S rRNA sequences obtained were then compared with sequences within the NCBI database using the Basic Local Alignment Search Tool (BLAST). Sequences with 98-100% identity to sequences deposited in the public domain databases were considered to be positive identification of taxa.

2.3 Detection of antimicrobial resistance and virulence genes

The DNA of all 59 isolates was analysed for the presence of antibiotic resistance genes including those for erythromycin, tetracycline and vancomycin. In addition, detection of the virulence associated genes of gelatinase, surface protein, cytolysin and aggregation substance was done based on a previous protocol (7) (Table 1).

Gene target(s)	*Primer*	*Oligonucleotide sequence* (5'-3')	*Product size (bp)*	Tm (°C)
16S RNA	27f 1492r	GTGCTGCAGAGAGTTTGATCCTGGCTCAG CACGGATCCTACGGGTACCTTGTTACGACTT	1164	54
Erm(A)	ERMA1 ERMA2	TCTAAAAAGCATGTAAAAGAA CTTCGATAGTTTATTAATATTAGT	645	50
Erm(B)	ERMB1 ERMB2	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTC	639	52
tet(M)	TETM1 TETM2	AGTTTTAGCTCATGTTGATG TCCGACTATTTAGACGACGG	1862	55
tet(L)	TETL1 TETL2	CCTGCGAGTACAAACTGG TCAAGGTAACCAGCCAAC	1209	55
vanA	VANA1 VANA2	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	50
vanB	VANB	ATGGGAAGCCGATAGTC	635	52

	VANB2	GATTTGCTTCCTCGACC		
vanC1	VANC1-1 VANC1-2	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	50
vanC2/3	VANC2/3-1 VANC2/3-2	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	439	50
gelE	GELE1 GELE2	ACGCATTGCTTTTCCATC ACCCCGTATCATTGGTTT	419	51
esp	ESP1 ESP2	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCA	932	58
cylB	CYLB1 CYLB2	ATTCCTACCTATGTTCTGTTA AATAAACTCTTCTTTTCCAAC	843	50
prgB, asa, aspI	AGG1 AGG2	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	1555	52
asa373	ASA373F ASA373R	GGACGCACGTACACAAAGCTACC TGGGTGTGATTCCGCTGTA	619	58

Table 1. PCR primers used to detect antimicrobial resistance genes of erythromycin (*erm(A)* and *erm(B)*), Tetracycline (*tet(M)* and *tet(L)*), Vancomycin (*vanA, vanB, vanC1, vanC2/3*) and virulence-associated genes of *E. faecalis (*gelatinase *(gel(E)),* enterococcal surface protein (*esp*), cytolysin (*cylB*), aggregation substance (*asa373, prgB, asa, aspI*).

Amplicons were analysed by gel electrophoresis at 60 V/cm^2 in 1% (w/v) agarose. A 100-bp DNA ladder (Promega) served as the molecular weight standard. A positive control was extracted DNA from a VRE isolate and the negative control was ultrapure water. Gels were stained with safe view dye (NBS biologicals, Cambridgeshire, UK) and PCR products were visualised under UV light illumination.

2.4 RAPD-PCR analysis

Random Amplified Polymorphic DNA analysis with arbitrary nucleotide sequences of M13R2 (5‘GGAAACAGCTATGACCATGA3’) and M13 (5‘GAGGGTGGCGGTTCT3’) was applied to the 59 *E. faecalis* isolates. All reactions were performed in a 50-µL final reaction volume containing 1 µM of each primer, 25 µL of PCR Master Mix (Promega, Madison, USA) and 2 µL of total DNA template.

According to the protocol described by Andrighetto et al; 2001(17), PCR included 35 thermal cycles of 94°C for 45s, 40°C for 30s and 72°C for 2 min. Gel electrophoresis of the amplicons was performed in a 1.5% (w/v) agarose gel at 60 V/cm^2. Amplicons were stained with safe view dye (NBS biologicals, Cambridgeshire, UK) and subsequently detected by UV light transillumination. A 100-bp DNA ladder (Promega) served as the molecular weight standard.

Gelcompar software (APPLIED MATHS, c2012) was used to analyse the agarose gel electrophoresis images. Jaccard correlation coefficient and cluster analysis by the unweighted pair group method with arithmetic average (UPGMA) was used to compare banding patterns.

3- RESULTS

3.1 Detection of antimicrobial resistance and virulence genes of *E. faecalis*

Detection of antimicrobial resistance and virulence genes for all the 59 isolates is presented in Figure 1. Among the Brazilian endodontic isolates, PCR products for tetracycline resistance were detected. The *tetM* gene was detected in 12 isolates, whilst *tetL* was present in 4 isolates, representing 60% and 20% of all the isolates, respectively. The genes *ermA, ermB, vanA, vanB, vanC1* and *vanC2/3* were not found in any of these isolates. PCR for specific virulence genes showed that all 20 isolates (100%) were positive for gelatinase (*gelE*), whilst an enteroccocal surface protein (*esp*) gene was detected for 13 isolates (65%), and aggregation substance genes (*prgB, asa, aspI)* was found in 18 isolates (90%). All isolates were negative for the *asa373* (aggregation substance) gene and the cytolysin gene, *cylB*. As observed for Brazilian isolates, PCR products for tetracycline resistance were detected in the oral UK isolates. The *tetM* gene was found in 7 isolates, whilst *tetL* was present in 1 isolate, representing 70% and 10% of the isolates, respectively. Unlike the Brazilian isolates, in this group,

one isolate was positive for the *ermB* gene, representing 10% of oral isolates from the UK and two isolates were positive for the gene *cylB* (cytolysin), representing 20%. The *ermA, vanA, vanB,* and *vanC1 vanC2/3* genes were not detected. PCR for specific virulence genes showed that all 10 isolates (100%) were positive for *gelE*. The *esp* gene was detected in 5 isolates (50%), and *agg* was found in 8 isolates (80%). These results were similar to those of the Brazilian isolates. Japanese hospital isolates had a similar genetic profile to oral isolates, but had a higher prevalence of *ermB* and *cylB* and no *tetL* was detected. All VRE isolates were positive for *gelE, esp, agg, vanA, ermB* and *tetM*, 95% were positive for *cylB* and 17% were positive for *tetL*. All isolates were negative for *ermA, vanB, vanC1* and *vanC2/3* genes.

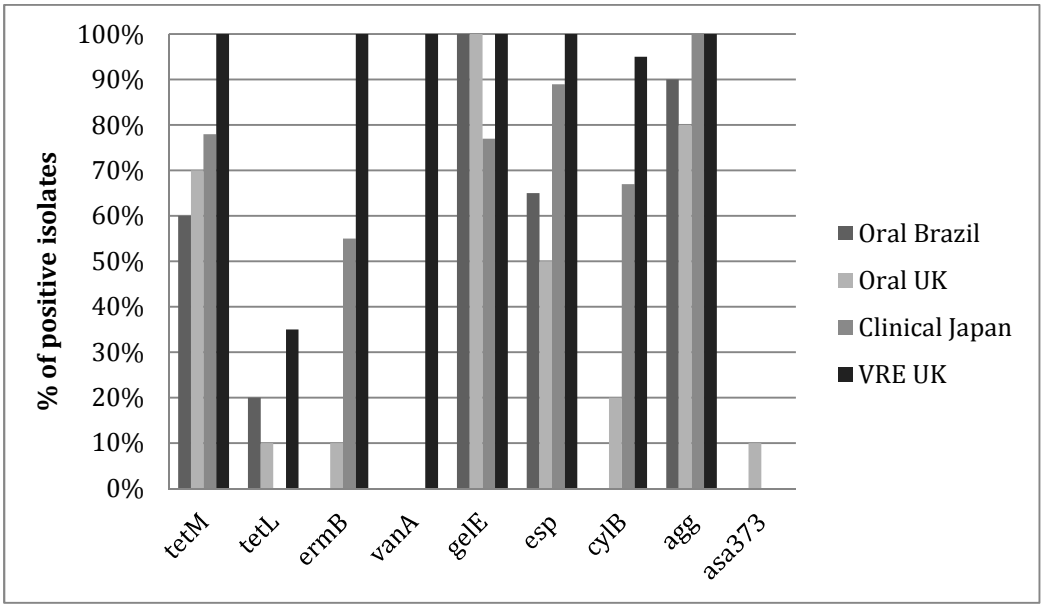

Figure 1. Detection (%) of antimicrobial resistance and virulence genes amongst *Enterococcus faecalis* isolates

3.2 RAPD-PCR analysis

RAPD-PCR analysis using the primer M13R2 distinguished 24 RAPD types. All 20 VRE isolates yielded the same fingerprint and thus formed a single cluster at a

similarity level of 100%.

RAPD-PCR analysis using the primer M13 revealed 47 RAPD types. Figures 2 and 3 show the UPGMA dendrogram obtained with primers M13R2 and M13, respectively.

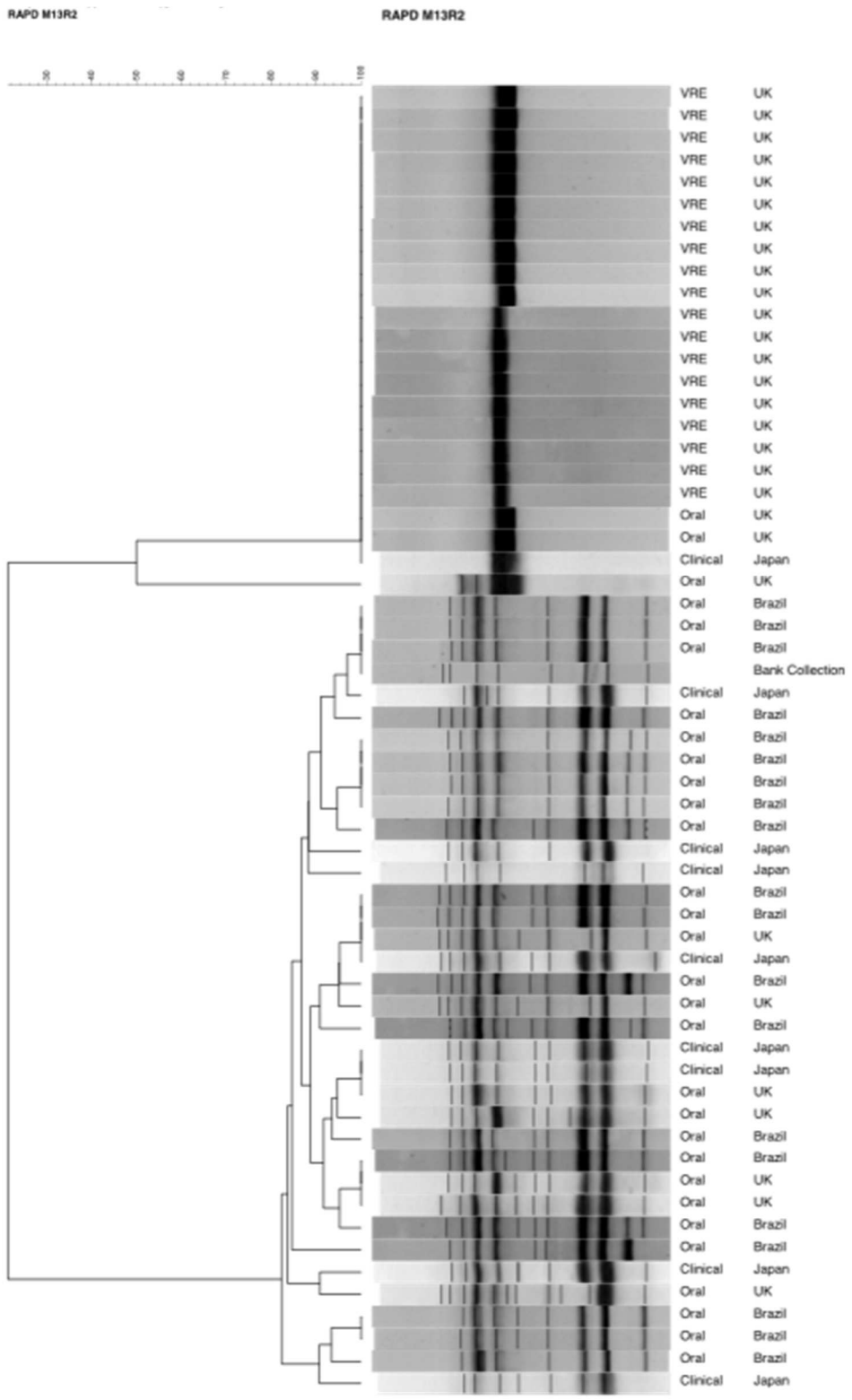

Figure 2- Dendrogram of RAPD profiles obtained with primers M13R2 for *E. faecalis* isolates (created using UPGMA)

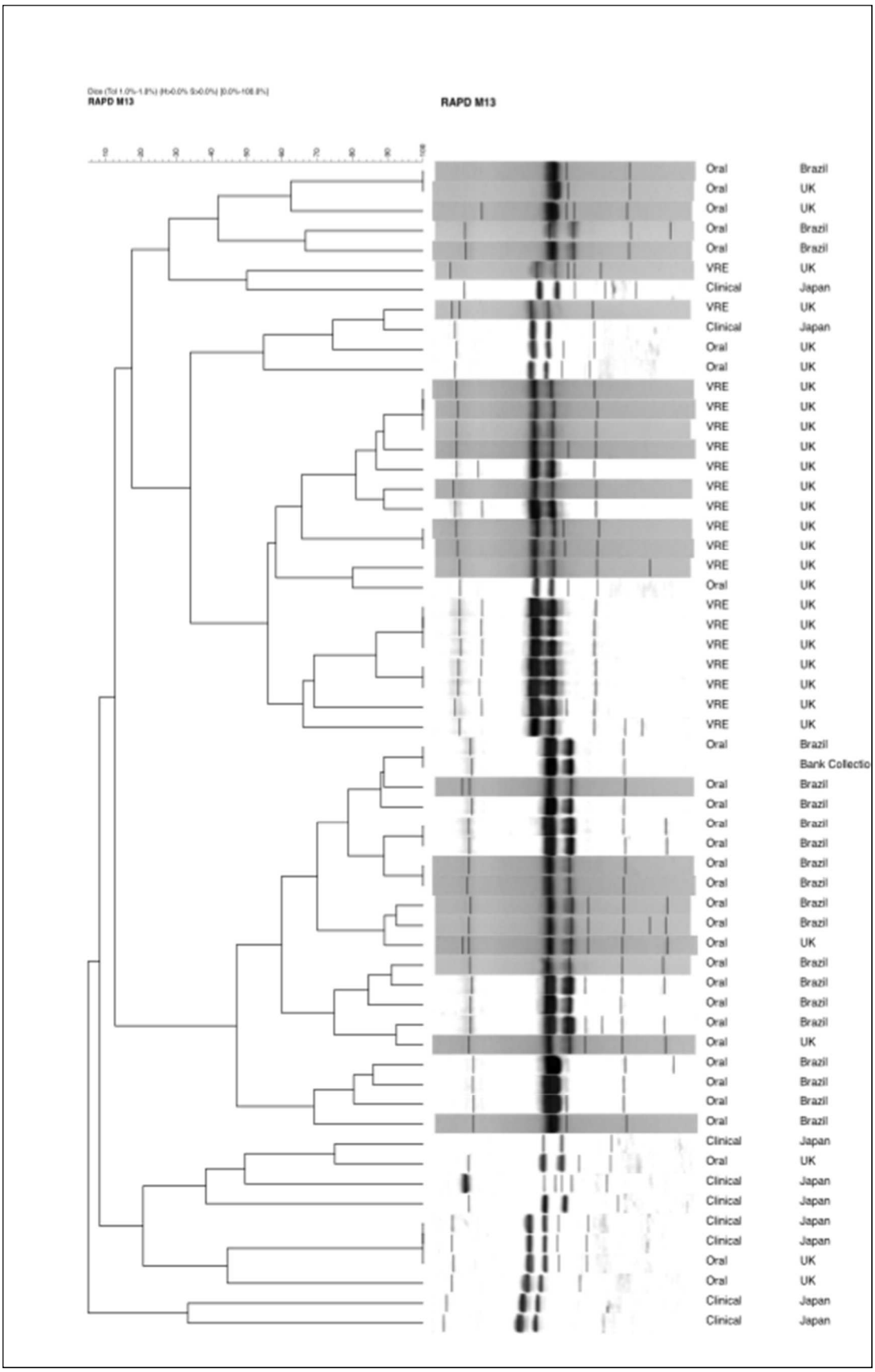

Figure 3 - Dendrogram of RAPD profiles obtained with primers M13 for *E. faecalis* isolates (created using UPGMA)

4- DISCUSSION

The view that enterococci are not widely regarded as normal components of the oral microflora needs to be reviewed. These microorganisms are considered transient colonisers of the oral cavity and have been isolated from a range of oral sites including the mucosa, carious lesions, chronic periodontitis and endodontic infections, especially in persistent cases (4, 5, 7, 14, 18). Importantly, the source and the role of enterococci in the pathogenesis of endodontic infections remains unclear (6,11,19). Recently, Ayre et al, 2018 (20) showed in an *ex vivo* polymicrobial pulpal infection model that *E. faecalis* pathogenicity in pulpitis was linked to its greater ability to attach to the pulpal vasculature. Wang et al, 2012 (10), showed that the prevalence of *E. faecalis* in the root canal system was associated with its occurrence in saliva, suggesting that enterococci may enter the root canal system during or after endodontic treatment. However, Vidana et al, (11) analysed the genetic relationship between *E. faecalis* from root canals and isolates from different host sources using pulsed-field gel electrophoresis and showed that isolates from the root canals were not related to those from the normal gastrointestinal microflora. None of these patients were documented to have enterococci in their saliva. Furthermore, Zhu et al, (5), also found differences in PFGE profiles when comparing *E. faecalis* isolates from root canals and saliva within a given individual. To date, an endogenous source of these isolates has not been confirmed. Foodborne transmission of *E. faecalis* was suggested by Zehnder and Guggenheim, (12) in a narrative review that explored the potential reasons for the high occurrence of enterococci in filled root canals. Vidana et al (1) investigated the possibility of foodborne transmission but could not provide evidence, based on a lack of similarity in both virulence and antibiotic susceptibility profiles between isolates from food and root

canal. Also, the possibility of nosocomial transmission in dental clinics was highlighted due to the robust nature of the microorganisms, which have been shown to resist desiccation for months (21). It was concluded that the potential for nosocomial transmission of enterococci from environmental surfaces in dental surgeries although small, required further investigation (13).

Given the difficulties in finding a single origin for endodontic *E. faecalis* we hypothesised that oral strains could have close genomic fingerprints to those reported for isolates from the hospital environment and hospitalised patients, also carrying PAIs, multiple virulence genes and antimicrobial resistance determinants. To evaluate this, we undertook molecular profiling using a well-established approach and analysed the results in the context of virulence genes expressed within a collection of isolates from a range of clinical sources and geographical areas. This would provide a better understanding of the involvement of different reservoirs in the emergence and spread of virulent clones, as well as be indicative of genetic relatedness between them.

Enterococcus faecalis PAI encodes proteins of many different functions and virulence, including the enterococcal surface protein (Esp). This protein, encoded by the esp gene, has not been reported as occurring in any other genetic element. As a result, many previous studies have considered that *E. faecalis* isolates positive for esp will also be positive for PAI presence (3). Our results showed a high prevalence of esp gene in Brazilian endodontic isolates (65%), which was a little higher than for oral isolates from UK (50%). This incidence of *esp* for oral isolates was similar to values previously reported, which ranged from 40% to 61% (3, 19, 22). Considering the presence of *esp* in oral isolates and its association with *E. faecalis* PAI, the potential occurrence of virulent clones colonising the oral cavity is not unrealistic. Furthermore, it has been shown that pathogenic genes can be transferred in the microenvironment of

the root canal via endodontic *E. faecalis* (23).

Comparison of the genetic profiles between *E. faecalis* isolated from primary endodontic infections in Brazil with oral isolates from UK does not indicate whether there is a difference in actual expression of the genes detected. Among the isolates from the UK, one endodontic isolate showed a wider profile of virulence, presenting with *ermB* and *tetM* and *clyB* encoding the cytolysin, a putative gene that has not been found in previous studies of root canals infections and is associated to increased virulence (3, 6, 7).

Comparison of the genetic profiles between oral isolates with non-oral Japanese isolates showed a more pathogenic profile in the latter. This was because the non-oral Japanese isolates frequently possessed all virulence genes with the exception of *gelE*. Furthermore, the Japanese isolates also had genes encoding cytolysin and resistance to erythromycin, with an incidence of 67% and 56%, respectively. Regarding tetracycline resistance genes, the Japanese isolates exhibited a higher *tetM* frequency.

As expected, genetic profile analysis of the VRE hospital environment group showed that the studied genes were more frequently encountered. This is in agreement with Kuch et al. 2012 (24), who found resistance level to vancomycin and other antibiotics was much higher in isolates from clinical infections of hospitalised patients compared to community isolates.

Phylogenetic analysis using RAPD-PCR is distinct from classical PCR in its use of a single primer that is not directed or targeted to any known sequence of the bacterial genome and is arbitrarily designed. RAPD-PCR is a well-accepted and reliable tool for differentiation and identification of enterococci. By comparing standardised RAPD and PFGE protocols with computerised methods involving band positioning, it has been found that typing of VRE by PFGE and RAPD generates highly congruent DNA

fingerprint clusters (25).

In dental research, very few studies have used RAPD-PCR for molecular typing of enterococci. Al Badah et al (15) used RAPD-PCR to study 21 *E. faecalis* isolates, including 19 from primary endodontic infections and 2 from retreated root canals isolated in Saudi patients. Cluster analysis of the RAPD-PCR profiles discriminated five different genotypes. In this current study, the RAPD-PCR analysis revealed large genetic variability among the isolates, although it was also noted that several were genetically indistinguishable, suggesting a possible relationship, although they were isolated from different sites and distant geographical origins. This variation in the RAPD genotypes allowed verification of heterogeneity regarding geographical origin and isolation site, except for the VRE. In this study, use of the primer M13R2 was less discriminatory than when primer M13 was used, with the latter generating 47 RAPD genotypes. Clustering of the different studied groups was evident by RAPD, including the VRE group.

5- CONCLUSION

In summary, RAPD-PCR used to genotype *Enterococcus* has potential for broad application in oral microbiology epidemiology. Genetic analysis of 59 isolates demonstrated that although there was a higher frequency of certain genes isolated in *E. faecalis* hospital groups, all groups of different geographical origin and isolation sites had these genes. This finding confirms our hypothesis that these isolates have common characteristics and important virulence potential for pathogenesis. Clearly, we cannot exclude the possibility that oral enterococcal infections may serve as potential reservoirs of pathogenic enterococcal clones. There also remains a necessity for further investigation into their pathogenesis. Questions remain about the effectiveness of root

canal treatment, with the concept that bacteria may reside in the root canal causing inflammation and potentially leading to systemic disease. Environmental pressures in root canals may be responsible for selection of more resistant strains and ones possessing virulence determinants.

Our study demonstrated a significantly high incidence of antimicrobial resistance genes for tetracycline, which is an antibiotic still used in local treatment of endodontic infections. This reinforces the debate about local use of this and other antibiotics for oral infection. Prospective studies in this area are required, particularly in relation to the possession and expression of virulence factors in the root canal environment, to better inform management strategies.

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