Detection of *Enterococcus faecalis* and *Candida albicans* in previously root-filled teeth in a population of Gujarat with polymerase chain reaction

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Abstract

Background: Micro-organisms are the primary causative agents of endodontic infections. Phenotype based procedures for bacterial identification has certain drawbacks especially, when investigating the microbiota of root-filled teeth. Thus, more sensitive methods like Polymerase chain reaction (PCR) can provide results that are more accurate and reliable for the microbial prevalence in the root filled teeth. **Aim:** In this study, we have investigated twenty symptomatic root-filled teeth with chronic apical periodontitis for the prevalence of *Enterococcus faecalis* and *Candida albicans* in the root filled teeth associated with symptomatic cases with or without periradicular lesions. **Materials and Methods:** Microbiological samples were taken from the canals immediately after removal of previous gutta percha cones using aseptic techniques. After removal of root canal filling, samples were obtained with paper points placed in the canal. Paper points were transferred to a cryotube containing "Tris EDTA" buffer and immediately frozen at –20°C. **Results:** By PCR amplification of the samples using taxon specific primers, *E. faecalis* was found to be prevalent species, detected in 65% of the cases and *C. albicans* was detected in 35% of cases. **Conclusion:** The results of the study shows that geographical influence and dietary factors might have some role to play in the prevalence of the species like *C. albicans* and presence of *E. faecalis* confirming the assertion of previous culture-dependent and independent approaches for the microbiological survey of root filled teeth.

Keywords: *Candida albicans*, *Enterococcus faecalis*, polymerase chain reaction, root filled teeth

Introduction

Studies investigating the intraradicular microbiota associated with treatment failures have reported the occurrence of micro-organisms in 44-100% of the cases with variation in the type of species and its occurrence with different geographical locations.[1-9] Collection of samples from root filled teeth is difficult due to limitations imposed by physical constraints of root canal and presence of root canal filling materials. Moreover, micro-organisms that resists root canal treatment exists in low numbers in the root canal or can be located in areas of difficult access to paper points used for sampling. Thus, a low number of microbial cells may be collected and if less sensitive methods are used for identification these organisms pass unnoticed and their prevalence in failed cases can be over shadowed. Polymerase chain reaction (PCR) has advantages of being more sensitive than culture and possibly less affected by chemical factors such as remnants of medications that can enter a sample and inhibit the microbial growth in the laboratory.[10] These methods can also detect the occurrence of fastidious or even uncultivable bacterial species in endodontic infections including the cases of secondary persistent infections associated with failed endodontic therapy. Epidemiologic studies investigating the microbiota of root canal treated teeth have revealed that *Enterococcus faecalis* is the most commonly detected species associated with treatment failures irrespective of the method used for identification. Also, the presence of *Candida albicans* has recently been studied in retreatment cases.[11] The purpose of this study was to investigate the prevalence of *E. faecalis* and *C. albicans* in the root filled teeth associated with symptomatic cases with or without periradicular lesions in a population of Gujarat.

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Materials and Methods

The study was approved by the university ethical committee. All patients provided with the written informed consent before being recruited in the study. Patients with symptomatic previously root filled teeth were divided into two groups in the study,

i. Those that had endodontically treated teeth and were symptomatic with radiographic evidence of periradicular periodontitis requiring endodontic retreatment.

ii. Those that had endodontically treated tooth that were symptomatic requiring retreatment without radiographic evidence of apical pathosis and presence of periapical radiolucency or periodontal ligament widening.

Inclusion criteria were: Root canal treatment done atleast 1 year back (Single rooted or multi rooted teeth), poor quality of obturation, coronal leakage, missed canal, age between 18 years and 40 years, no antibiotics taken within a pre-period of 1 month [Table 1].

Following variables were registered for each patient:
Pre-operative signs and symptoms: Defined as moderate to severe pain to percussion or palpation or any flare ups where the patient returns for unscheduled appointments with symptoms
Quality of previous filling (acceptable obturation was 2 mm short of the apex, 0.5 mm beyond apex, with voids no more than 1 mm)
Quality of the coronal restoration
History of Diabetes Mellitus.
Negative control-distilled water

Positive control were the Strains of *E. faecalis* (American Type Culture Collection 29212) and *C. albicans* (ATCC 10231) obtained from microbiological culture (Microbiologics-Himedia, Ahmedabad).

Samples were collected using strict asepsis. Each tooth was cleaned with pumice and isolated with a rubber dam. The tooth and the surrounding field was then cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite solution. This has been demonstrated to be better than iodine for decontamination of the tooth surface before sampling for PCR. Coronal restorations were removed using sterile burs with saline irrigation and the operative field, including the pulp chamber was then swabbed with 2.5% sodium hypochlorite. The root canal fillings were removed without the use of solvents, and a small amount of sterile saline solution was deposited into the canal without overflowing. Gates Glidden drills and K-type files were used for removal of the filling material. The working length was established 1 mm short of the radiographic apex. After removal of filling material, the root canal walls were then gently filed to generate dentin chips. Thereafter, 2 to 3 sequential paper points were placed to the working length and used to soak up the fluid in the canal. Each paper point was retained in position for 1 min, transferred to a cryotube containing "TE" buffer (10 mM Tris–HC1, 0.1 mM EDTA. pH 7.6), and immediately frozen at -20°C. In case of multi rooted teeth the samples were obtained from one canal (distal in lower molars or palatal in upper molars) and in case of periradicular lesion sample was taken from that root.

DNA extraction

The root canal samples in TE buffer were thawed to 37°C for

Table 1: Sampling procedures

Sample no	Age/sex	Symptoms	Level of root filling (mm)	Radiographic changes	Time since obturation (years)	Other	Tooth
1	56/F	+	5	PR	2	M.C.	26
2	18/F	++	4	PR	2	Unfilled	16
3	42/F	+	2	PR	2	S.C.	36
4	30/M	++++	3	PR	3	swel	37
5	37/F	++	4	PR	3	–	46
6	35/F	++	6	PR	10	–	16
7	42/F	+++	>6	PDL	5	–	46
8	45/M	++++	5	PR	3	swel	37
9	22/F	++	3	PR	2	–	47
10	30/F	+++	2	PR	4	S.C.	16
11	45/M	++	2	PR	4	–	22
12	46/F	++	5	PDL	4	–	37
13	32/M	++	5	PR	3	Unfilled-D	36
14	20/M	++	4	PDL	2	–	37
15	38/F	+++	2	PR	10	Resorption, S.C.	11,12, 21
16	22/F	+++	2	PR	5	swel	16
17	36/M	++	4	PR	3	–	46
18	41/M	++	1	PR	2	–	36
19	34/M	+++	5	PR	1+	–	46
20	41/F	+	3	PDL	5	–	34

PR: Periapical radiolucency; PDL: Periodontal ligament widening; SC: Single cone; MC: Missed canal; swell: Swelling

10 min and homogenized by vortex mixing for 1 min. The paper points were removed, and the microbial suspension was washed 3 times with 200 µl of sterile Milli-Q water by centrifugation for 2 min at 2500 × *g*. After the final wash,pellets were resuspended in 200 µl of Milli-Q water, boiled for 10 min in a water bath,quickly chilled on ice for 5 min, and centrifuged at 9000 × g at 4°C to remove unbroken cells and large debris. The supernatant was collected and used as template for PCR amplification.

PCR assay

Aliquots of 5 µl of supernatant (cell lysate) from clinical samples or 1 µl of the reference strain were amplified. The PCR reactions used to assess the occurrence of *C. albicans* and *E. faecalis* were performed in a 50 µl of reaction mixture containing 0.5 µM of each specific primer [Table 2],[12] 5 µl of 10 × PCR buffer (Bangalore Genei Pvt. Ltd, Bangalore, India), 1.5 U of *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India), 2 mM $MgCl_2$ and 0.2 mM concentration of each deoxyribonucleoside triphosphate (deoxyadenosine triphosphate, deoxythymidine triphosphate: Biotools).

Sterile Milli-Q water replacing microbial DNA template was used in each PCR procedure as a negative control, and DNA extract of the targeted species was used as a positive control. PCR reaction was carried out in Master Cycler (Applied Biosystem, U.S.A) as per the cycling parameters mentioned in Table 3.[12]

PCR amplicons were analyzed by agarose gel electrophoresis in 1.5% agarose gel run at 4 V/cm in Tris-Borate-EDTA buffer. The gel was stained for 15 min with 0.5 µg/ml Ethidium bromide and visualized under ultraviolet light. Positive reactions were determined by the presence of bands of the expected sizes. A 100 bp DNA ladder (Bangalore Genei Pvt. Ltd, India) served as the molecular size standard.

Table 2: Polymerase chain reaction primers used in this study

Organisms	Primers [5'-3']	Amplicon size (bp)
Enterococcus faecalis	*F-GCC GGT GAC GAC GCT CCA AGA GCT G* R-CCG TCA GGG GAC GTT CAG	310
Candida albicans	*F-GCC GGT GAC GAC GCT CCA AGA GCT G* R-CCG TGT TCA ATT GGG TAT CTC AAG GTC	158

PCR: Polymerase chain reaction

Results

Of the target species, *E. faecalis* was the most prevalent detected in 13 out of 20 cases (65%) cases and *C. albicans* was present in 7 (35%) cases. These species were mostly not present together except in 2 cases. On PCR amplification of *E. faecalis* and *C. albicans* with taxon specific primers yielded amplicons of predicted size 310 bp [Figures 1 and 2] and 158 bp [Figures 3 and 4] as analyzed by agarose gel electrophoresis. Similarly, clinical samples that were positive for the target taxa also showed only single band of expected size as of positive controls. As expected, amplification was not seen in negative controls, inferring the absence of any discrepancies in the results. The detection limit of the PCR assay used in this study was 10-100 cells as determined by amplification of serial 10-fold dilutions of templates prepared from reference DNA. Taking into account the dilution factor dictated by the use of aliquots of the whole original sample, the detection limit for the various test taxa was then 400-4000 cells.

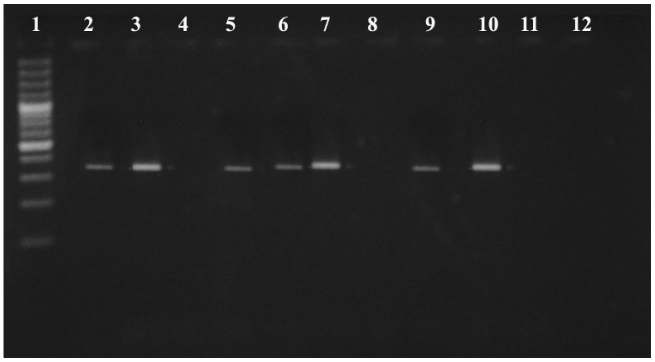

Figure 1: Amplicons of *Enterococcus faecalis* of predicted size 310

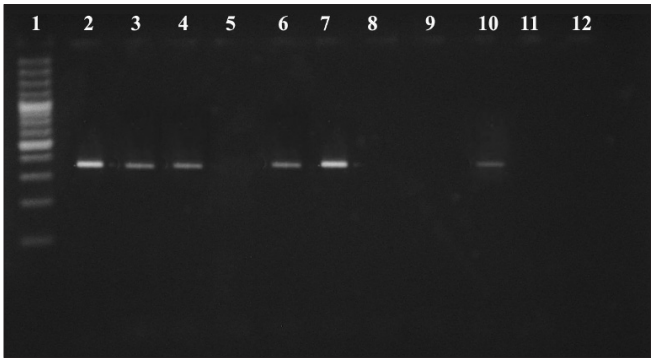

Figure 2: Amplicons of *Enterococcus faecalis* of predicted size 310

Table 3: Cycling parameters for polymerase chain reaction reactions

Organisms	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
Enterococcus faecalis	95°C-2 min	95°C-0:30 min	60°C-1 min	72°C-1 min	72°C-2 min	36
Candida albicans	95°C-2 min	95°C-1 min	55°C-0:30 min	72°C-1 min	72°C-10 min	35

PCR: Polymerase chain reaction

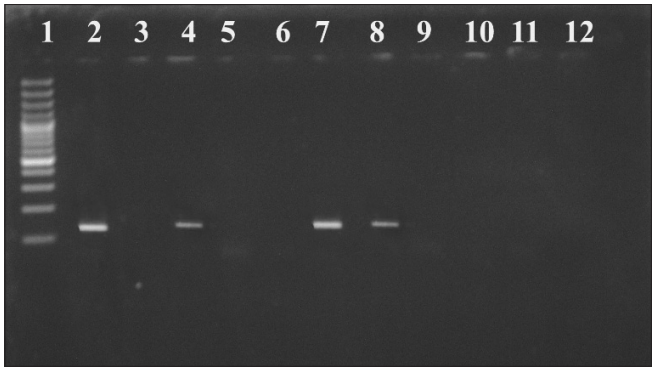

Figure 3: Amplicons of *Candida albicans* of predicted size 158

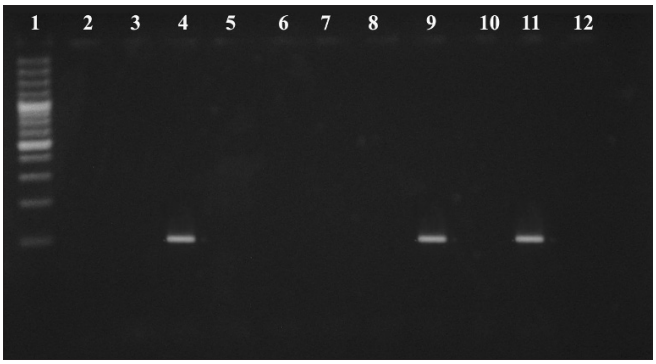

Figure 4: Amplicons of *Candida albicans* of predicted size 158

Discussion

Over the last 10 years, several studies[1-9] have investigated the microbiota associated with treatment failures. Apart from one study,[4] all of them have unanimously found *E. faecalis* to be the most prevalent micro-organism isolated or detected in those cases. In the present study, *C. albicans* was found in 35% of the root filled teeth. This high prevalence is similar to the culture based study by Cheung and Ho[4] in Chinese patients, which failed to isolate *E. faecalis* from the investigated teeth. This study was performed in India and it might have a geographical influence showing higher number of yeasts as dietary factors have a role to play. High frequency of yeasts has been reported in retreatment cases when culture methods have been used for detection of these microbes suggesting a pathogenic role for this organism.[2,13] High prevalence of enteric bacteria and yeasts in root-filled teeth with chronic apical periodontitis has been well established. Baumgartner *et al*.,[14] used a probe and PCR techniques to demonstrate yeasts in 21% of teeth with apical periodontitis yeasts may be more frequently recovered from root canals of obturated teeth in which treatment has failed. Yeasts may gain access to the root canal during treatment via contamination through their presence in saliva with coranally unsealed teeth. Culture studies shows that 10% of primary cases involved the presence of *Candida* spp,[15] compared to 15% and 3% of root filled teeth with chronic periodontitis in Lithuania[6] and Sweden,[2] respectively, and 7% of culture positive samples in 967 samples from persistent endodontic infections in Finland.[16] Using PCR, *C. albicans* was detected in 21% of infected root canals[14] and 9% of cases of failed endodontic therapy.[11] Regional variations may also exist in the frequency and Colony Forming Unit values for yeasts, although this is unknown. *C. albicans* form biofilm which it might use to survive a toxic flux in these inorganic areas of teeth. The biofilms of *C. albicans* forms thin layers of cells [decorated with small amounts of extracellular polymers] in microtitre plates as well as on the polystyrene surface of the Calgary Biofilm Device. However, *C. albicans* and *C. tropicalis* biofilms are highly resistant and/or tolerant to toxic metal species.[17] Immature biofilms that consist of little more than adherent layers of cells, as well as those in the early stages of growth, have elevated resistance to antibiotics and metals relative to planktonic cells. Possible factors include the ability to adhere to dental tissues, the utilization of hyphae to penetrate into dental tubules, identify breaks on surface or between cells through which, hyphae can penetrate ("Thigmotropism"). Secretion of protease that allows degradation of human proteins may also be a contributory factor. Secreted aspartyl proteinases (Sap Proteins) at tissue lesions sites have been shown to directly contribute to *C. albicans* pathogenicity. The Sap protein digest molecules for nutritional purposes, disrupts host cell membranes for adhesion and invasion, and also target immune cells for digestion to avoid killing, for example, by acting as cytolysins in macrophages after *Candida* has been phagocytosed. The ability to switch between phenotypes to allow adaptation to different ecological conditions and between the yeast form and hyphae form may also contribute to pathogenicity although, the mechanisms are not clear. Consistent with these observations the surface attachment might trigger the physiological transition to a multidrug and multimetal resistant and/or tolerant state for *Candida* spp.[16] *C. albicans* and some other *Candida* species have been shown to be even more resistant to calcium hydroxide *in vitro* than *E. faecalis*.[13]

Although, information gathered in the present study was based on a relatively small number of samples, prevalence of *E. faecalis* was 65% which not significantly different in comparison with that observed in various studies in different geographical locations[3,5-7,12] using the same identification method suggesting that this species can play a relevant role in the etiology of persistent periradicular lesions. *E. faecalis* possesses an array of virulence traits that may play a role in the pathogenesis of periradicular diseases.[18] Possibilities exist that more virulent clones of *E. faecalis* may be present in teeth with periradicular diseases. Furthermore, host responses varies hence, a definitive conclusion about the occurrence of *E. faecalis* in persistant periradicular lesion cannot be drawn. This conspicuous predominance of *E. faecalis* does not appear to be influenced by geographical locations, although differences in prevalence values have been reported.[12] However, whether *E. faecalis* is the major pathogen associated with treatment failures or a mere survivor that takes advantage of its ability to endure adverse conditions within obturated root canals has not been established.

Thus, the possibility exists that during chemomechanical preparation, the most prevalent anaerobic bacteria species are

more likely to be eliminated other than *E. faecalis*, which has been demonstrated to be resistant to intracanal antimicrobial procedures and medications, including calcium hydroxide.[19] Considering that *E. faecalis* can be found as a single species in obturated root canals associated with recalcitrant periradicular lesions, one may surmise that this species is able to maintain or induce periradicular inflammation by itself and therefore can be an endodontic pathogen and the major causative factor associated with post-treatment periradicular lesions. Promising results regarding effectiveness against *E. faecalis* have been reported by laboratory studies using combinations of calcium hydroxide with other substances, such as camphorated para monochlorophenol or chlorhexidine or chlorhexidine alone in gel form or in a slow-release device. It must be pointed out that all cases examined in this study were poorly obturated, and this can favour the establishment or persistence of fastidious anaerobic bacteria.[5,7]

One of the possible limitations of the PCR assay used in this study is that it does not provide quantitative results. As a consequence, it is difficult to ascertain whether detected species occurred in large enough numbers to participate in the infectious process. The high sensitivity of the assay allows it to detect microbial species that are present even in low numbers in the sample. However, this does not diminish the importance of the results because, as far as we are concerned, the microbial load necessary for a periradicular lesion to be induced is still unknown and may vary depending on several aspects, such as clonal type virulence, presence of support species in the mixed consortium [synergism or additism], and host resistance. Although, these factors are not elucidated, it would obviously be prudent to use the method with the highest sensitivity to detect all species colonizing the root canal. Moreover, even species that are present in small numbers can participate in the infectious process, because they may support species or possibly highly virulent clonal types, which even in low numbers can play a pathogenic role.

In brief, the results of this study strengthen the assertion that intraradicular infections are the main cause of endodontic failures. It is noteworthy that all examined samples contained organisms. Either *C. albicans* or *E. faecalis* occurred in the canals sampled at times together and sometimes individually. Future studies should be directed at expanding the patient population, its clinical characteristics, and the diversity of micro-organisms identified in root canals with necrotic pulp. The present study concurs with most previous studies using culture-dependent or culture-independent approaches for the microbiological survey of root filled teeth in that *C. albicans* and *E. faecalis* were one of the most prevalent species detected. *E. faecalis* being a common member of the microbiota associated with such cases, regardless of geographical location. However, the presence of organisms like *C. albicans* cannot be ruled out and the geographical influence and dietary factors might have some role to play in the prevalence of this particular species.

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