

PCR and Culture Analysis of Enterococcus Faecalis from Retreatment Cases in Indian Population

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Abstract: Enterococcus faecalis has long been mentioned in increased frequency with regard to teeth with post-treatment disease (PTD). The prominence of E. faecalis in root-filled teeth with apical periodontitis has made it a focus of attention as an etiological factor of PTD. To evaluate and compare bacterial culture and species-specific polymerase chain reaction for the detection of enterococcus faecalis in retreatment cases with apical periodontitis in rural Indian population. A total of 80 samples were obtained from 40 patients, divided into 2 groups and analyzed by the two methods. The samples were grown under anaerobic conditions during culture and the determination of the microbial species was based on biochemical tests. DNA was extracted and analyzed for PCR using species-specific primer. The association of microbiological findings with clinical features was investigated. Enterococcus faecalis was isolated from 46.5% of the selected teeth. Culture and PCR detected the test species in 25% and 67.5% of the samples. PCR was significantly more effective than culture in detecting this bacterial species. E. faecalis was clearly proven as the causative factor of apical periodontitis. Therefore, combining molecular and culture technique is probably the best approach available to provide comprehensive and a panoptic information about the microflora associated with endodontic infections.

Keywords: Enterococcus faecalis, apical periodontitis, polymerase chain reaction, DNA, Culture techniques.

INTRODUCTION

Apical periodontitis is caused by microorganisms colonizing the root canal system. A single treated canal associated with post-treatment disease can harbor a density of 10^2 to 10^5 bacterial cells and 1-2 species per canal [1].

Molander *et al* have shown that the root canal flora from failed cases is primarily gram-positive bacteria [2]. Hence, the ultimate goal of endodontic treatment of teeth with apical periodontitis is eradication of bacteria from the root canal system [3]. Cultivation based identification approaches have several limitations, one of the reasons being bacterial unculturability due to the lack of essential nutrients [4]. Novel culture-independent methods for microbial identification has been used to investigate the microbial diversity, without the biases of culture. Moreover, NanoDrop 1000 spectrophotometer quantifies and assesses the purity of DNA prior to amplification [5].

In previous studies, *Enterococcus faecalis* has been proven to be predominantly associated in retreatment cases with apical periodontitis [6]. It can be attributed to the various virulence factors present, such as adherence to host cells [7]. The polymicrobial

bacterial profile in apical periodontitis significantly varies from subject to subject [8]. Differences are even more pronounced in samples from individuals of different countries [9]. Hence, the scarcity of data from the Indian population regarding this analysis adds to the significance of this study.

MATERIALS AND METHODS

Subjects and Methods

Before the beginning of the study, the patients gave their written informed consent to the study protocol, which was reviewed and approved by the ethics committee. Eighty clinical samples from forty subjects (men and women, 18-75years of age), with radiographic evidence of apical periodontitis were collected for the study. The samples were divided into two groups.

Group1 (40 Nos): Detection of *Enterococcus faecalis* in patients of retreatment cases with apical periodontitis, using culture method.

Group2 (40 Nos): Detection of *Enterococcus faecalis* in patients of retreatment cases with apical periodontitis, using species-specific PCR method. Medical and dental histories as well as patient's clinical and radiographic evaluation obtained were charted in the case sheets.

Sampling procedures

The teeth were isolated using a rubber dam and the surrounding field were cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite (NaOCl) solution. Endodontic access was completed with a sterile high-speed carbide bur. Coronal gutta-percha was removed by means of sterile Gates-Glidden burs, and the apical material was retrieved by using K-type or Hedstrom files, or both. The filling material removed from the canals was transferred to nutrient agar and the cryotubes. The root canal contents were absorbed into at least four paper points. Each paper point was retained in position for one minute. Forty paper points of Group I were transferred to reduced transport medium (RTM) solution for cultivation and forty paper points of Group II were transferred to RTM solution and immediately frozen at -20°C for PCR amplification.

After vortexing, 50µl of sample was plated onto MacConkey's Agar culture media. *Enterococcus faecalis* was incubated under aerobic condition in an incubator at 37°C for 48 hours. Biochemical analysis for the identification of bacteria was noted. The DNA extracted from the clinical isolates was amplified by PCR respectively. Reference DNA from several species was also extracted to serve as positive control for the taxon-specific primers used or to evaluate the specificity of the primers. The DNA concentrations in clinical samples and the concentrations of the reference DNA were determined by NanoDrop 1000 spectrophotometer.

Nano Drop Analysis

The DNA concentrations in clinical samples and the concentrations of the reference DNA were determined by NanoDrop 1000 spectrophotometer. The optical density (OD) was measured at different wave lengths: 230 nm, 260 nm, 280 nm, and 320 nm. The OD_{260/280} ratio is used as indicator for DNA purity. Serial 10-fold dilutions of known concentration of reference DNA of the target species were processed to determine PCR assay sensitivity. The lowest DNA concentration that resulted in a positive PCR product was regarded as indicative of the sensitivity of the assay.

PCR Amplification

The PCR reaction used to assess the occurrence of all target taxa, was performed in 50 µl of reaction mixture containing 38 µl sterile distilled water, 5 µl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1.0% Triton X 100), 3 µl 25 mM MgCl₂, 1 µl 10 mM dNTPs (10 mM each dATP, dTTP, dGTP, dCTP), 1 µl 20 µM forward primer, 1 µl 20 µM reverse primer, 0.2-1 µl Taq polymerase. Negative controls consisting of ultrapure water instead of sample were included with each batch of samples analyzed. DNA amplification was performed in a thermal cycler system (Eppendorf Master Cycler). Amplicons were stored at -20°C. The amplification products were analyzed through the use of electrophoresis in a 1.5% agarose gel conducted at 4V/cm in Tris-borate EDTA buffer. The data collected was statistically analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The results were statistically evaluated using Student unpaired T test and Pearson chi-square test.

RESULTS AND DISCUSSION

Results

The following results were obtained while comparing both the techniques; cultivation (group 1) and PCR (group 2). The study group comprised of subjects from the Indian population of age ranged from 20 to 70 years (mean age = 32 years, SD= 10.83). More number of retreatment cases was seen in the age group of 20-25 years in molars. Of the 40 cases, 6 teeth were single rooted and 34 were multirooted.

The study clearly demonstrated that *E. faecalis* is the dominant species in retreatment cases of apical periodontitis in Indian population. *Enterococcus faecalis* was isolated from 46.5% of selected teeth from both groups.

Negative results were seen in 21.25% of teeth (17/80) in both the groups. All culture positive cases were also PCR positive.

E. faecalis was detected in 10 teeth (25%) in group I, while PCR procedures revealed the occurrence of the species in 27 teeth (67.5%) in group 2. PCR was significantly more effective than culture in detecting this bacterial species (p=0.016).

The clinical signs and symptoms present in the 40 patients were as follows: 27/40 cases with pain, 31/40 with tenderness on percussion, 10/40 cases with swelling, 9/40 with sinus tract, 28 cases with lesion size of about <2mm, 10 cases with lesions size ranging from 2-5mm and 2 cases with lesion size of about >5mm. most presented with poor restoration- 19/40, 4/40 cases with good restorations and 4/40 with open cavities. Upon radiographic examination, most root filled teeth had obturation about 3mm short of radiographic apex (22/40) while 2 cases presented with the apical limit 2mm short of radiographic apex. 37/40 cases were

poorly obturated canals while 3/40 had good root fillings.

E. faecalis was most frequently recovered from patients with pain, tenderness on percussion and lesion size of about 2mm in group 1- 7/27(25.9%), 8/31(25.8%) and 6/28 (21.4%) respectively and 20/27(74%), 23/31(79.3%) and 19/28 (67.8%) in group 2.

E. faecalis was identified in 4/12 (33.3%) and 10/12 (83.3%) cases showing periarticular bone destruction, in group 1 and group 2 respectively. *E. faecalis* was more associated with poorly restored teeth with 9/40 (22.5%) and 27/40(67.5%) found in group 1 and group 2 when compared to well-sealed restorations with 1/40 (2.5%) and 2/40 (5%) revealed in

group 1 and group 2 respectively. 2/9(22.2%) cases with sinus tract showed the presence of *E. faecalis* in group 1 and 6/9(66.6%) in group 2.

Considering the length of the treatment, *E. faecalis* was most frequently found from the time period of 3 years (18/40) with 8/18 (44.4%) samples being positive of the microorganism in group 1 and 10/18(55.5%) in group 2. *E. faecalis* was seen in cases with the apical limit short by at least 3mm(55%) and under filling with voids and under extension without apical seal, where 5/22(22.7%) of the target microorganism was obtained in group 1 and 15/22(68.18%) in group 2. Apparently there was no statistical significance seen in any of signs and symptoms with the association of *E. faecalis* in root filled teeth.

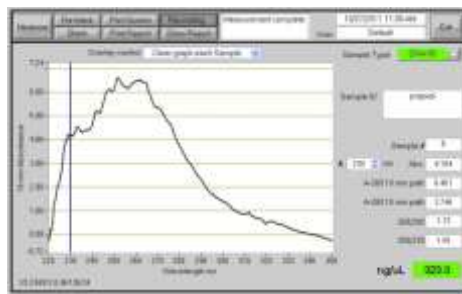
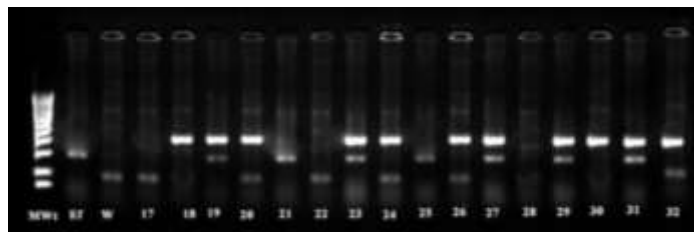
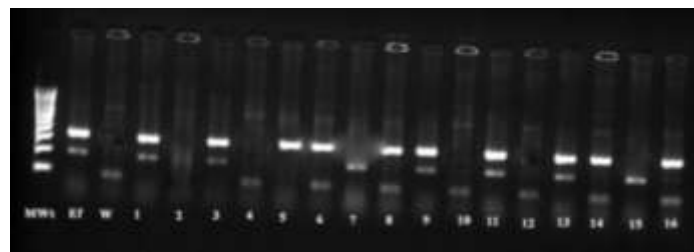
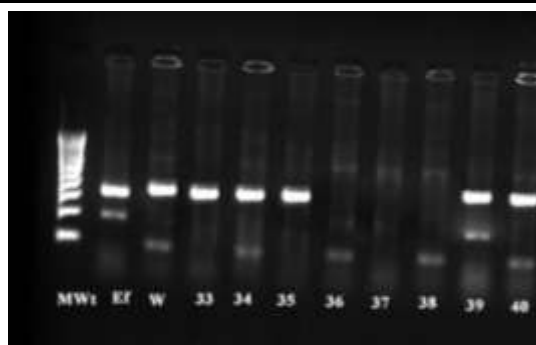


Fig-1: Graph obtained after nano drop analysis showing the purity of DNA

Table-1: PCR primers, with expected amplicon size and the rmocycling parameters used in the present study

Target	Sequence (5' to 3')	Size bp)	Amplification cycles
Enterococcus faecalis	GTT TAT GCC GCA TGG CAT AAG AG CCG TCA GGG GAC GTT CAG	310	36 cycles: 95°C 30 s, 60°C 1 min, 72°C 1 min





MWt- molecular weight, Ef- Enterococcus faecalis, W-water

Fig-2: Agarose gel pictures of Enterococcus faecalis

Table-2: Comparison of cultivation and PCR techniques for detection of Enterococcus faecalis in retreatment cases with apical periodontitis

			PCR (Group 2)		Total
			Yes	No	
CULTURE (Group 1)	Yes	Count	10	0	10
		%	34.5%	.0%	25.0%
	No	Count	19	11	30
		%	65.5%	100.0%	75.0%
Total		Count	29	11	40
		%	100.0%	100.0%	100.0%

Sensitivity =34.5% Specificity= 100 % Positive predictive value=100 % Negative predictive value= 36.7%. Chi-square test showed statistically significant association between PCR and Culture (p= 0.016)

DISCUSSION

Apical periodontitis is an inflammatory disease that affects the tissues surrounding the apical portion of the dental root and is primarily caused by microorganisms infecting the root canal [10]. Sundqvist *et al.* found that gram positive, facultative anaerobes predominated amongst the isolates from root filled teeth with apical periodontitis [2]. Persistent intraarticular or secondary infections are the major causes of the failure of root canal treatment [1].

For more than a century, cultivation has been the standard diagnostic test in infectious diseases. However, there are many possibilities for bacterial unculturability such as the impossibility of cultivating a large number of bacterial species and the difficulties in identifying many cultivable species [5]. Numerous derivatives in PCR technology have been developed since its inception. In an attempt to minimize test costs, we have attempted to use the species specific PCR.

In the past decade, the prominence of *E. faecalis* in root-filled teeth with apical periodontitis has made it a focus of attention as an etiological factor of PTD [9]. Marked incongruity in detection of *E. faecalis* in root samples examined by culture techniques and those examined by molecular based methods has been shown in previous studies, indicated that independent analysis of the same root canal sample by parallel culture and molecular techniques are indicated [1, 4, 7].

Since the bacterial profile in apical periodontitis significantly varies from subject to subject and due to the geographical differences [11], the aim of our study was to evaluate and compare bacterial culture and species-specific polymerase chain reaction for the detection of enterococcus faecalis in retreatment cases with apical periodontitis in rural Indian population.

More number of retreatment cases was seen in age group of 20-25 years. Frequency of molars was higher, that can be due to the presence of an unusual root or root canal morphologies associated with molars that have been recorded in several studies [12]

Our study verifies the fact that *E. faecalis* is the dominant species in retreatment cases of apical periodontitis in Indian population that is 46.5%. The high prevalence of *E. faecalis* in root-filled teeth is in accordance with previous studies.

E. faecalis was recovered from 10 of 40(25%) root canals examined by culture in group 1. The higher specificity of culture results in previous studies can be attributed to the additional experiments done during culture in the presence of excess sodium chloride (6.5%) and testing for hydrolysis of bile esculin. Although the sampling techniques and laboratory procedures used in this study have been shown to be highly effective, it is indeed a technique sensitive method. It is possible that some microorganisms present could have been lost, especially if the number of microorganisms present in the root canal was very low

or if they were present in areas such as anatomical branches and apical areas obliterated by previous treatment.

In group 2, 27 of the 40 cases were positive of *enterococcus faecalis* detected by PCR analysis thereby showing higher sensitivity over culture. Previous studies [10] using molecular methods found *E.faecalis* in 77%, 67% and 64% of the cases, which strongly support our results. These findings disagree with those reported by Rolphet *al.* who have not found *E.faecalis* in any of the canals investigated [13].

One possible reason for the more effective results by PCR is the ability of molecular methods to detect DNA from dead cells. However, it is highly unlikely that DNA from dead cells can remain intact in a complex background like the infected root canal. After cell death, the DNA molecule faces an onslaught of nucleases from other bacteria and fungi in the environment. Other chemical processes, such as oxidation and hydrolysis, can also contribute to DNA damage over time, causing an irreversible loss of nucleotide sequence information [8,11].

The key to good molecular biology results is proper DNA quantification and failure to get high quality data can sometimes be directly attributed to an incorrect estimate of the concentration of DNA template used. The amount of genomic DNA required for PCR ranges from 50 to 500 ng, with each amplification requiring fresh DNA as template [9]. Therefore the use of the Nano Drop spectrophotometer to measure both quantity and purity is beneficial in order to obtain better reporting.

E.faecalis was more associated with poorly restored teeth compared to well seal restorations. Thus, bacterial presence without lesions can be interpreted as a consequence of coronal exposure of root canal fillings to saliva. Studies have demonstrated that recontamination of the entire extent of treated canals can occur shortly after direct saliva challenge [11]. Moreover, in cases with the apical limit short by at least 3mm and under filling with voids and under extension without apical seal, *E.faecalis* was predominant. Our results was in line with studies demonstrated by Hancock *et al.* that stated canals that are not filled adequately and hence not cleaned adequately, render more bacteria-most likely because an environment similar to that of an untreated infected canal exists[2]. It can also be the result of bacteria present in the accessory canal, ramification, isthmuses, within the dentinal tubules, or associated or associated with the remnants of gutta-percha [8]. Hence, these bacteria may reside not only in the canal lumen but also may invade the dentinal tubules for more than 200 microns. *E. faecalis* may survive in the smear layer and in debris inside the root canal (and inside the lateral canals and dentinal tubules) and may be extremely difficult to

remove by irrigation and instrumentation [14]. Our study proved that not only the apical limit but also good coronal restoration is important for the successful outcome of an endodontic therapy [13].

E.faecalis was most frequently recovered from patients with pain and tenderness on percussion in both the groups. It may emerge as a result of a disturbed equilibrium between the host defense and the bacterial infection in an already established lesion, mostly from the action of planktonic bacteria such as *enterococcus faecalis*. 25% of the patients were associated with swelling and 21.4% of the cases were most commonly associated with lesion size of about 2mm. Previous studies had examined root filled teeth with persistent apical radiolucencies (considered secondary apical lesions) and found that *Enterococcus species* predominated [2]. This can be due to destruction, by localized inflammation, of the often extremely thin bone lamella between the sinus floor and the root apex which results in a local mucous membrane reaction in the form of membrane swellings. The etiology of apical periodontitis is an infection of the tissues in the root canal system and of the surrounding dentin, in some cases also of tissues outside the apical foramen or other portals of entry. Our study has corroborated the finding that *E.faecalis* is a causative factor apical periodontitis in retreatment cases by culture and PCR.

The teeth included in this study had been previously filled by guttapercha, which is a material that has considerable antibacterial activity. In previous studies, bacteria were isolated also with all other materials that had been used as root fillings. Obviously, this is an indication that the root filling materials gradually lose their antibacterial activity in the root canal to an extent that allows survival and even growth of bacteria. These bacteria must be ecologically strong in such a way that they can survive in the environment of incompletely filled root where the availability of nutrients may often be limited, compared with primary apical periodontitis.

E. faecalis was most frequently seen in cases that were treated at the time span of about 3 years. The length of treatment had no apparent influence on the occurrence of *E.faecalis*. However, there were no statistical significance between culture and PCR regarding the signs and symptoms.

We detected *E. faecalis* in 17 samples that were negative by cultivation, suggesting that the bacterium was in the VBNC (viable but nonculturable) form. In this state, bacteria escape detection by conventional culturing methods as they are unable to grow on either solid or liquid culturing media but are still alive, metabolically active and able to exert pathogenicity. VBNC cells can resume active growth when favorable environmental conditions are reestablished. Thus, viable *E. faecalis* entombed at the

time of root canal filling may provide a long term nidus for subsequent infection [11].

In our study, zinc oxide eugenol based sealers were used, which had shown statistically significant antimicrobial efficacy against *Enterococcus faecalis* in previous studies. This is a strong indication that rather than the previous treatment, it is the present ecological conditions in the incompletely filled root canal that have the key role in creating a selective ecological pressure to the composition of the infective flora.

Bacteria that resisted intracanal procedures and are present in the canal at the filling stage can influence the outcome of the endodontic treatment provided that they resist to treatment-induced disturbances in the ecology of bacterial community. *E. faecalis* has the capacity to produce cytolysin by quorum-sensing mechanism [7].

CONCLUSION

PCR was significantly more effective than culture in detecting this bacterial species. *E. faecalis* was clearly proven as the causative factor of apical periodontitis. Therefore, combining molecular and culture technique is probably the best approach available to provide comprehensive and a panoramic information about the microflora associated with endodontic infections.

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