

DETECTION OF *ENTEROCOCCUS FAECALIS* IN SYMPTOMATIC AND ASYMPTOMATIC ENDODONTIC INFECTIONS BY CULTURE AND PCR METHODBharati D. Deo^{1*}, Shashikala K.² and Kishore G. Bhat³¹Dept. of Conservative Dentistry Govt. Dental College and Research Institute, Bangalore/Bellary India.²Dept of Conservative Dentistry, DAPMRV Dental College, Bangalore, India.³Maratha Mandal's NGH Dental College and Research Institute, Belgaum, India.***Corresponding Author: Bharati D. Deo**

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ABSTRACT

Objective: The objective of the study was to detect *Enterococcus faecalis* from root canals of teeth with symptomatic and asymptomatic endodontic infections by using culture and polymerase chain reaction (PCR) methods. **Study design:** Microbial samples were collected from 31 symptomatic and 23 asymptomatic untreated teeth and from 5 symptomatic teeth with failing endodontic treatment. *E. faecalis* was isolated and detected by culture method and PCR using species specific primers targeting 16SrDNA. **Results:** *E. faecalis* was detected in 5.6 % in symptomatic and 21.7% in asymptomatic cases by culture and it was seen in 33.3% of symptomatic and 34.8% of asymptomatic cases by PCR method. The detection of *E. faecalis* between the groups was significantly different when culture method was used. It was not significant when PCR method was used. **Conclusion:** *E. faecalis* was detected significantly more frequently in asymptomatic group by culture method.. PCR method was more sensitive in detecting the target organisms than the culture method. However, no significant difference in the detection of *E. faecalis* was seen between symptomatic and asymptomatic endodontic infections when PCR method was used.

KEYWORDS: *E. faecalis*, culture, PCR, endodontic infections.**INTRODUCTION**

Endodontic infection is unique in nature as the root canal is a site where microorganisms are not previously present, unlike the other microbial diseases of the oral cavity like dental caries or periodontal disease. In the case of dental caries and periodontal disease, the microbial biofilm is previously present and the disease occurs when there is a change in the environmental conditions, whereas the infection of the root canal occurs where the environment is initially sterile.^[1] If the route of invasion is via caries, the bacteria in front of the carious process are the first to reach the pulp. Endodontic infections are polymicrobial in nature and has a strong predominance of strictly anaerobic bacteria is typical of primary endodontic infections, along with some facultative anaerobes such as Streptococci and *E. Faecalis*.^[2,3]

Enterococcus faecalis is capable of surviving in a starved environment for a long period of time and can invade the dentinal tubules and has the property of circumventing the antimicrobial effect of calcium hydroxide. It has several virulence factors such as aggregation substance, surface adhesions, gelatinase, and toxic cytolysin. This organism can play a serious role in the outcome of

endodontic treatment. Studies have reported *E. faecalis* to be present occasionally in cases of primary endodontic infections.^[4,5] In contrast, in cases of failed endodontic treatments, *E. faecalis* has been frequently isolated.^[6,7] Endodontic infection can be symptomatic or asymptomatic and the factors responsible for the development of symptoms are not completely understood. Early studies attempted to correlate some bacterial species with symptoms, but it has been demonstrated that the same species can also be found in asymptomatic cases.^[8,9] It seems that the appearance of symptoms might be a result of a complex mechanism, depending on diverse pathogen related and host-related factors. Currently, though the molecular methods are very prevalent and popular for the detection of microbes in the oral infections, yet culture analyses are not redundant There has been a significant focus recently on developing methods for the in-vitro cultivation of those species that were till recently refractory to culture.^[10] Over the years culture method has been used for the detection of *E. faecalis* from endodontic infections.^[11] Among molecular techniques, the polymerase chain reaction (PCR) technique has been widely used to detect bacteria in primary endodontic infections.^[12] The finding from microbiologic studies conducted at different parts

of the globe reveal that a given species that is very prevalent in root canals of patients from some geographic regions is not necessarily found in similar figures or even detected in samples from other locations.^[13] It has been suggested that genetic and environmental factors can influence the composition of oral microbiota particularly with regard to the periodontal pathogens.^[14,15] Similar variations were seen in microflora of endodontic infections.^[12]

Combining molecular and culture technique can provide comprehensive information about the microflora associated with endodontic infections. Hence an effort has been made in this study to identify *E.faecalis*, in symptomatic and asymptomatic primary endodontic infections in patients, by the culture and PCR method, targeting the 16Sr RNA gene, in the Indian population.

MATERIAL AND METHODS

Subject Population

After prior approval from the Institutional Ethical Board, of RV Dental College, Bangalore, and Government Dental College and Research Institute, Bangalore, Karnataka, India, 59 subjects were selected for the study. All the subjects were referred for endodontic treatment / retreatment. 54 subjects were of primary endodontic infection and 5 subjects required retreatment. A written informed consent was obtained from the selected subjects before sample collection. 38 male and 21 female subjects, between the age group of 17 to 76 years participated in the study. Subjects with systemic diseases like diabetes mellitus, cardio-vascular diseases, immune-compromised status and those with periodontal pockets and advanced bone loss as well as subjects on prior antibiotic therapy for 2 months were not included in the study. Thirty-six subjects had symptoms of spontaneous or provoked pain exacerbated by mastication or mild tenderness to percussion and twenty-three subjects were asymptomatic.

Microbial Sampling

Strict asepsis was followed during sample collection. After administration of local anaesthesia, the tooth was isolated with rubber dam. The tooth and the dam along with the clamp were scrubbed with 1% sodium hypochlorite for one minute followed by inactivation with 5% sodium thiosulphate. Caries and/or existing restorations, if present were removed and then the cavity was wiped with a sterile cotton pellet slightly wet with 1% sodium hypochlorite and then inactivated with 5% sodium thiosulphate. The pulp chamber was then accessed with a sterile bur, using sterile saline solution for irrigation. A sterile no. 15 k file was introduced in the canal up to 3mm. short of the apex and the canals briefly instrumented. If purulent or serous fluid was present in the canal, this was directly sampled with the paper points, (placed in the canal for 30 seconds). Otherwise sterile saline was deposited in the canal and a K file depending on the size of the canal was introduced

to the working length and canals briefly instrumented and later the paper points were used to take the sample. The collected samples were placed in a sterile plastic vial, of 2 ml. capacity, containing 1. ml. of RTF (Reduced Transport Fluid) and were transferred to the laboratory.

Microbial Isolation

Culture

For the culture, the following procedure was followed:- Sample (1ml) in the transport medium (RTF) was first vortexed then it was diluted with RTF at 1:10 proportion and inoculated on Blood Agar as the culture medium. The rest of the sample was utilized for PCR. The plates were incubated at 37 C in 5-10% CO₂ jar for 48-72 hrs. After the completion of incubation the plates were removed and the colony characters of the organisms were noted and the colony count was done for quantification. The count of the colony was multiplied by 1000 to get the CFU/ml. The organisms were confirmed by Gram staining and following key biochemicals. tests :- Indole, Catalase, enzyme detection, Nitrate Reductase test and Sugar fermentation tests.

PCR Procedures

From the remaining (0.9 ml.) sample, DNA was extracted by modified Proteinase K method and was refrigerated at -20°C for use in PCR experiment which was carried out later.

A premixture was prepared and aliquoted into each tube which was thin walled. The premix (AMPLIQON RED 2X Mastermix) contained Tris-HCL pH 8.5, (NH₄)₂SO₄, 3mM MgCl₂, 0.2% Tween 20, 0.4mM of each dNTP, 0.2 units/μl ampliqon Taq DNA Polymerase and an inert red dye and stabilizer components in a final volume of 20 μl/aliquot. The mastermix was thawed, then gently vortexed and briefly centrifuged. The PCR tube was placed on ice and ampliqon red master mix: 10 μl, *E.faecalis*(Forward primer): 0.5 μl (10 pmole), *E.faecalis* (Reverse primer): 0.5 μl (10 pmole), Template DNA: 2 μl (< 1 μg/ reaction) and water was added to make final volume to 20 μl. The Samples were gently vortexed and spinned down and the PCR tubes were placed in the thermal cycler (Applied Biosystem, USA).

PCR primers were used which is specific to *E.faecalis*
Forward primer: CCG AGT GCT TGC ACT CAA TTG G
Reverse primer: CTC TTA TGC CAT GCG GCA TAA AC

The amplification products were analysed by 2% agarose gel electrophoresis, was stained with ethidium bromide. The PCR products were visualized under UV light.

The organisms detected by culture and the DNA detected by PCR are divided in two groups, Group-1 Endodontic Infections with symptoms and Group-2, Endodontic Infections without symptoms are presented in Table-1 and Table-2 Percentage of overall cases in which microbes detected by culture method and DNA by PCR

method are calculated are presented in Table-3, and group-wise detection of organisms by both methods is presented in Table-4 and the graphic representation of the same is depicted in Fig.-1.

As there were only five cases of endodontic treatment failure, and all belonged to the symptomatic group, a separate group was not made for these cases.

STATISTICAL ANALYSIS

The detection of target organisms was recorded as a percentage of cases examined in symptomatic and asymptomatic groups by culture and PCR method. Using the Standard Normal Variable Test (Gaussian's method) it was proved that detection of *E.faecalis* by PCR was significantly higher as compared to culture method. Detection of *E faecalis* in asymptomatic group was significantly high ($p<0.001$) as compared to the symptomatic group by using culture technique. Difference in the detection of *E.faecalis* between

symptomatic and asymptomatic group was statistically not significant.

RESULTS

Overall *E.faecalis* was detected in 7/59 (11.9%) cases by culture method and in 20/59 (33.9%) cases by PCR method. In Group-1, Endodontic infections with symptoms, *E.faecalis* was detected in 2/36 (5.6%), cases by culture method and in 12//36 (33.3%) cases by PCR method. In the same group, 5 cases were of failed endodontic treatment, out of these, *E.faecalis* was not detected in any case by the culture method-(0%), but in 2/5 (40%) cases by PCR method. In Group-2, Endodontic infections without symptoms, *E. faecalis* was detected in 5/23 (21.7%), cases by culture method and in 8/23(34.8%) cases by PCR method. There were five cases of failed endodontic treatment in the symptomatic group. In these cases it was observed that *E.faecalis* was not detected in any of the cases (0/5) by culture method and it was observed in 40% (2/5) of the cases by PCR.

TABLE-1: Detection of *E.faecalis* in endodontic infections with symptoms.

Sample No.	CFU/ml (Culture Method)	DNA (PCR Method)
A-1	-	-
A-3	-	-
A-5	-	-
A-6	-	-
A-7	-	-
A-8	-	-
A-9	-	-
A-11	-	Detected
A-12	-	Detected
A-15	-	-
A-16	-	-
A-21	-	Detected
A-26 (R)*	-	-
A-27 (R)*	-	-
A-30	-	-
A-31	-	Detected
A-32	-	Detected
A-33	-	Detected
A-34 (R)*	-	-
A-37 (R)*	-	Detected
A-39	-	Detected
A-41	-	-
G-4	260,000	Detected
G-5	-	Detected
G-6	-	-
G-8	32,000	Detected
G-9	-	-
G-12	-	-
G-13	-	-
G-14	-	-
G-16	-	-
G-17	-	-

G-18	-	-
G-20	-	-
G-23 (R)*	-	Detected

R*- Failed Endodontic Treatment Cases

TABLE-2: Detection of *E.faecalis* in endodontic infections without symptoms.

Sample	CFU/ml (Culture Method)	DNA (PCR Method)
A- 2	-	-
A- 4	-	-
A- 10	-	-
A- 13	-	-
A- 14	-	-
A-17	-	-
A-23	-	-
A-24	-	Detected
A-25	-	Detected
A-28	-	-
A-35	-	-
A-36	-	-
A-38	-	Detected
A-40	-	-
G-1	160,000	Detected
G-2	80,000	Detected
G-3	260,000	Detected
G-7	300,000	Detected
G-11	-	-
G-15	-	-
G-19	-	-
G-21	-	-
G-22	200,000	Detected

TABLE -3: Detection of *E.faecalis* in percentage of cases of endodontic infections by.

Culture Method	PCR Method
7/59 (11.9%)	20/59 (33.9%)

TABLE-4: Detection of *E.faecalis* in Percentage, of cases of endodontic infection by.

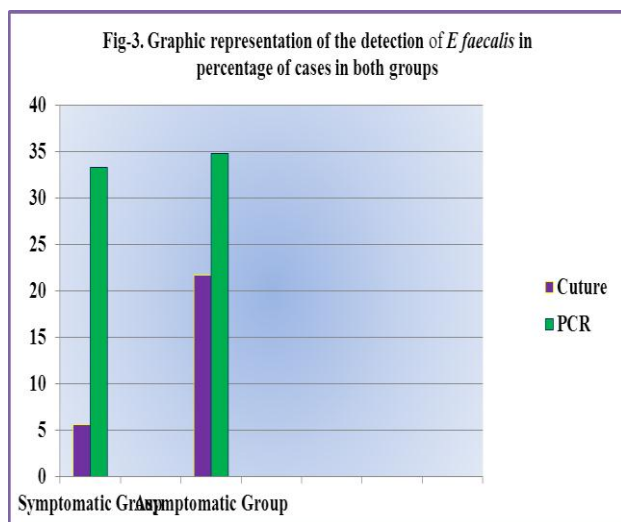
Group	Culture	PCR
Symptomatic Group (36 Cases)	5.6%	33.3%
Asymptomatic Group (23 Cases)	21.7%	34.8%



Fig-1: E.faecalis colonies on agar plates by culture method.



Fig-2: Image of agarose gel electrophoresis showing PCR products from root canal samples



DISCUSSION

Culture versus PCR

In the present study *E. faecalis* was detected in 11.9% (7/59) of all the samples (both Group-1 and Group-2),

from cases of endodontic infections as examined by culture method and in 33.9% (20/59) of the samples from cases of endodontic infections by PCR method. In Group-1, the symptomatic cases, *E. faecalis* was detected in 5.6% of cases by culture method versus 33.3% of cases by PCR method. Whereas, in Group-2, the asymptomatic group, *E. faecalis* was detected in 21.7 % of cases, by culture method versus 34.8% of cases, by PCR technique. This shows the higher sensitivity of the PCR techniques over the culture method in the detection of *E. faecalis* in endodontic infections. This is in accordance with the previous studies,^[16,17] where it was seen that PCR is more sensitive than culturing in the detection of bacteria associated with endodontic infections. Moreover *E. faecalis* might be in the viable but not cultivable (VBNC) state.^[18] The results of this study are in accordance with those of the previous studies.

Symptomatic versus Asymptomatic cases

It was seen in this study that in Group-1, the symptomatic group, *E. faecalis* was detected in 5.6% of cases by culture method and in 33.3% of cases by PCR method. Whereas in Group-2, the asymptomatic group, *E. faecalis* was detected in 21.7 % of cases, by culture method and 34.8% of cases, by PCR method. Hence it was seen that by both the methods the *E. faecalis* was detected more frequently in asymptomatic cases than in the symptomatic cases. In a previous study,^[19] the incidence 11.5% in asymptomatic teeth versus 3.7% in symptomatic teeth was reported. The results from another study,^[20] showed that *E. faecalis* has been seen in 10.5% of asymptomatic cases and 9.09% of symptomatic cases. Though the difference was not statistically significant, it was seen that *E. faecalis* was detected in more of asymptomatic cases. Our results are in accordance with this finding.

Amongst the symptomatic cases of endodontic infections, there were five cases where the treatment had failed. In these cases it was observed that *E. faecalis* was not detected in any of the cases (0/5) by culture method and it was observed in 40% (2/5) of the cases by PCR. In the primary endodontic infections *E. faecalis* was detected in 13% (7/54) cases, using culture method and 33.3% (18/54) cases, using PCR method. In the primary endodontic infections, the organism. has been detected by Rocas et al^[19] and Fouad et al,^[21] using 16S rDNA primers, at 18% and 8% respectively. Ozbek et al,^[22] using Real Time PCR detected the organism in 25% of cases. Sassone et al,^[23] using checkerboard DNA-DNA hybridization, detected the organism in 89 % of cases. Gomes et al,^[24] reported the detection of the organism in 4% of cases of necrotic pulps by culture method and 82% of cases by PCR method. The results from various studies differ in their detection rate. Earlier studies,^[25,26] reported nil or very low numbers in untreated canal, where culture method was followed and the later studies reported 82% detection where molecular techniques were followed. This underlines the sensitivity of the

molecular methods over the culture method, as well as the sensitivity amongst various molecular methods. Our results also fall in the range of those reported by previous studies.

In cases of secondary root canal infections, in our study, *E. faecalis* was not detected, by culture method and was detected in 40% of cases by PCR method. Rolph et al,^[27] did not detect the organism in their study using culture and PCR. It has been suggested that culture technique may fail to detect the bacteria in canals of root filled teeth, where the number of microorganisms can be low and /or the number of microbial cells can be lost during the removal of previous obturating material.^[28] As a consequence the number of cells in the sample may be lesser than the detection rate of culture method Fouad et al,^[29] using molecular technique, detected *E. faecalis* in 22% of the teeth where endodontic treatment was unsuccessful. Previous studies,^[22,24,28,30] using molecular methods have detected the organism in 76%, 74%, 77% and 64% of the secondary infections cases respectively. Sanhueza et al,^[31] in the failed root canal treatment cases, of Chilean population, showed a presence of 70% *Enterococcus spp.*, by culture, out of these 98% were *E. faecalis* as detected by PCR. A wide variation in the detection rate of this organism may be due to the difference in the molecular methods used, the status of the coronal restoration, and due to the differences in geographic locations of the cases. In our study using culture and PCR it was seen that *E. faecalis* is present in both symptomatic as well as in asymptomatic cases as well as in the cases referred for retreatment (where it was detected only by PCR technique), though the cases referred for endodontic retreatment were only five in number.

Anderson et al,^[32] reported that the oral isolates of *E. faecalis* had the highest percentage of virulence genes as well as extracellular enzymes and the capacity to form biofilms. They suggested that the oral cavity as a reservoir of virulent, antibiotic resistant, *E. faecalis* strains. A study by Komiyama et al,^[33] conducted on the Brazilian cohort, concurred with the above findings and reported that the adult and the elderly population have an increasing degree of presence of *E. faecalis* in the oral cavity.

A study on the biological status of *E. faecalis* in the VNBC state by Jiang et al,^[34] showed that the organisms in the VNBC state, had intact cell membranes and could adhere to collagen fibre type-1 and to dentin. Since this organism can evade chemomechanical procedures, invade the dentinal tubules and develop resistance to antibiotics, cases where it is detected in primary endodontic infections possibly pose a risk of secondary infection and treatment failure.

CONCLUSION

In the Indian population, *Enterococcus faecalis* was detected in symptomatic as well as asymptomatic

endodontic infections. There was a significantly higher rate of detection by PCR than by culture. In our study *E. faecalis* was detected in significantly higher frequency in asymptomatic cases than in the symptomatic cases, by the culture method. By PCR technique, though the detection in asymptomatic group was higher than the symptomatic group, the difference was not statistically significant.

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