



DIAGNOSTIC ACCURACY OF REAL- TIME PCR METHOD IN DETECTION OF CANDIDA ALBICANS IN INFECTED ROOT CANAL

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Abstract

This observational cross-sectional analytical study was conducted in the Department of Conservative Dentistry and Endodontics of Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh to detect candida albicans in root canal samples from endodontic infection. First, samples of infected root canal were taken from 50 patients who required root canal treatment. The carious lesion and coronal restoration were inactivated, the damaged tooth was isolated, and was decontaminated. Without using a chemical solvent, the root canal filling was removed. A radiograph was used to measure and confirm the canal's length. The microbiology lab received the paper points containing samples for identification of candida albicans by using two diagnostic methods culture and real-time PCR method assessing accuracy of real-time PCR. Candida albicans was isolated in 22 (44%) by real-time PCR on the other hand 10 (20%) by culture. Candida albicans is the most frequent fungus in root canals forming biofilms, regularly found in primary endodontic infections than in persistent infections, in which this yeast is resistant to irrigants and causes endodontic failure.

Key words: Candida albicans, Infected root canal, Real-time PCR, culture.

1. Introduction

The main cause of peri-radicular inflammatory disorders is endodontic infection, sometimes referred to as polymicrobial infection of the dental root canal system.¹ Candida albicans is by far the main

causative agent of oral candidiasis and accounts for up to 95% of cases. Although considered a pathogen, *C. albicans* is a ubiquitous commensal organism that commonly colonizes the oral mucosa. In fact, up to 80% of the general population are carriers.² *Candida albicans* is the most frequent fungus in root canals forming biofilms, regularly found in primary endodontic infections than in persistent infections, in which this yeast is resistant to irrigants and causes endodontic failure.³

An important consideration in endodontic treatment is the elimination of microorganisms, including fungi. *Candida albicans* (CA) plays an important role in endodontic treatment failure as the most important fungus isolated from the root canal system.⁴ *C. albicans* is the species most frequently isolated in endodontic infections, its prevalence in persistent infections is higher than in primary infections.⁵ The main supply of *C. albicans* in the body is found in the gastrointestinal tract and the development of infections occurs due to dysbiosis of the residential microbiota, immune dysfunction and damage to the muco-intestinal barrier.⁶ A small number of *Candida* species are part of the normal microbial flora of human mucosal surfaces and can give rise to opportunistic infections when host defenses are impaired. This pathogen is by far the most prevalent commensal *Candida* species.⁷ *Candida albicans* is the most frequently isolated fungus in endodontic root canal infections. Although it is recognized by cells in the dental pulp and peri-radicular tissue that elicit immune responses, it evades host defenses and causes cell death. This fungus then binds to tooth dentin, forms biofilms and invades dentinal tubules to resist intracanal disinfectants and endodontic treatments.⁸ It is understood that the majority of pulpal and peri-radicular diseases are caused by microorganisms. Although fungi have also been linked to infected root canals, bacteria have been the subject of the majority of research.⁹ Infected root canals may include a microbial environment that includes fungi, spirochetes, and bacteria. In several research, fungus from endodontic infections have been seen or grown.⁹ *Candida albicans*, a polymorphic fungus, is a component of the human microbiome. Most people have *candida albicans* as a permanent, unharmed commensal. However, under specific conditions, *candida albicans* can produce infections ranging from superficial skin infections to fatal systemic infections.¹⁰ *Candida* spp. were discovered infected tooth canals with a prevalence ranging from 0.5% to 55%.¹¹

There are numerous ways to find *candida albicans*, including polymerase chain reaction (PCR), electron microscopy, and culture. By using culture, molecular techniques, and in situ electron microscopy, it has been documented that *candida albicans* can grow in infected root canals.¹² The detection of fungi in diseased root canals has been subject to intrinsic limitations in earlier research utilizing microscopy or culturing methods.⁹ Polymerase chain reaction (PCR), as a vast diagnostic method, does not have the restrictions stated above and has the capability of recognizing even one copy of the explored DNA targets from clinical microbiologic samples.¹³ PCR in particular has seen increased use for *candida* detection due to its quickness, simplicity, specificity, sensitivity, and dependability.¹⁴

2. Methods

This observational cross-sectional analytical study during the period of 12 months (September 2021 to August 2022) was conducted jointly in the Department of Conservative Dentistry and Endodontics and the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Shahbag, Dhaka, Bangladesh.

Participants with infected root canal due to irreversible pulpitis, pulp necrosis, periapical abscess and persistent sinus tract were included for this study. A total of 50 participant's samples from infected root canal were collected for *Candida albicans*. On the basis of clinical and radiographic evaluations, the existence of a periapical radiolucent lesion that was either persistent or emergent were required for inclusion. In order to rule out confounding factors, patients with reinfection, systemic fungal infection, or taking antifungal treatment were excluded from the study. The individual data collecting sheet contained informed consent about each patient including case history, clinical examination findings, radiological assessment, microbiological analysis report, clinical signs and symptoms, as well as the quantity of root canal per tooth were noted.

Each patient's root canals were sampled and were cultivated for candida albicans in the culture laboratory and molecular analysis by Real-time PCR under stringent aseptic condition and also within biological safety cabinet.

For multi-rooted teeth, sampling was done in canals that were connected to exudation or periapical lesions. If all of the roots had lesion, the broader channel was chosen. Following mouth cleaning and rubber dam isolation, the tooth's crown and surrounding area were irrigated with 30% hydrogen peroxide, and 2.5% sodium hypochlorite solution¹⁵ was used to decontaminate the area for 30 seconds before being neutralized by 5% sodium thiosulfate solution¹⁶

Tooth was cleaned with pumice and isolated with a rubber dam. Teeth that could not be fully isolated with a rubber dam were excluded from the study. A gingival barrier (Opal Dam Light Cured Gingival Barrier, Ultradent) was used between the teeth and the rubber dam for each case. The tooth and surrounding field were cleaned with 35% hydrogen peroxide and decontaminated with a 5% sodium hypochlorite solution. After disinfection, the coronal restorations were removed. After completion of the endodontic access with a sterile high-speed carbide bur, the tooth, clamp, and adjacent rubber dam were once again disinfected with 5% NaOCl and then inactivated with sodium thiosulphate to avoid interference with the bacteriological sampling.

Using sterile small number k file and an electronic apex locator, the canal length was measured. A radiograph was then performed to confirm that the working length was accurate. After the filling material was removed, the canal was instrumented within 0.5–1.0 mm of the identified apex, and the root canal walls were softly filed to produce dentine chips. Afterwards, sterile paper points were placed into the canal, with each left for 60 seconds for absorbing all the fluids present within canals. These paper points (2% taper) were then transferred to autoclaved screw capped tube containing 3 mL of Brain Heart Infusion (BHI) broth (Bio-Rad). Sabouraud Dextrose Agar (SDA) media (Himedia Ltd.) was inoculated by samples taken from infected root canals. 0.1 ml of sample containing transport media disseminated with sterile platinum wire loop following streak plate method in sterilized 90 mm petri dishes after the prepared media had been sterilized at 121°C under 15 lbs for 15 minutes.

48 hours of aerobic incubation at 37 °C, petri plates were checked for the visible presence of whitish colored, raised, smooth and pasty colonies indicating the growth of fungi. Macroscopic inspection of the colonies revealed 0.5 to 1 mm tiny elevated colonies with whitish coloration. Identification of candida albicans was done by doing germ tube test. A small amount of colony of candida albicans was inoculated into a sterile autoclaved test tube containing 3 ml of human serum. Then the suspension was incubated at 37 °C for 2 hours. After incubation, a drop of suspension was examined under a microscope. Germ tubes raised as filamentous parallel cell wall extensions from the mother yeast cell. Extensions were 4 times the length of the cell and one half the widths, without constriction between the growing hyphal cell wall and mother yeast cell. The extension represented a beginning of a true hyphal element. Both constricted and non-constricted filaments may be seen in the GTT (Germ tube test) of *C. albicans*. The GTT was considered positive as any non-constricted filaments were identified. For microscopic examination, smears were prepared by placing a drop of Candida colony which was inoculated in 0.5 ml of human serum in the centre of glass slide and covered by cover slip and seen under light microscope by 40× magnification. Gram staining was done followed by the slides moving across the flame three to four times for fixing. The smear was stained with crystal violet for one minute, and then washed with tap water to do the gram staining. Similar to this, two minutes of Gram's iodine application were followed by a tap-water rinse. Then, drop by drop, 70% ethanol and 30% acetone were added. The smear was then counterstained for 45 seconds with 10% diluted carbolfuchsin before being removed once more with tap water. It was examined under a microscope at a magnification of 40 and then under a lens made of oil emulsion at a magnification of 100 after being dried with air for 3 to 5 minutes. It revealed gram positive for candida albicans.

Real-time PCR laboratory procedure:

All samples containing bottles were frozen at -20°C ¹⁷ before analysis with Real-time PCR. DNA was extracted using Bosphore DNA extraction spin kit (manufacturer: Anatolia genetworks, Istanbul-Turkey) which was based on the silica membrane column separation method following steps: Before using DNA extraction kit, 1.1 ml of PK(Proteinase K) storage buffer was added to Lysozyme and Proteinase-K (10mg/ml) solutions and stored at 4°C after resuspension. Lysozyme was supplied in lyophilized form. 20 ml of sterile dH_2O was added to Lysozyme and resuspended by vortexing.

Biological samples were spun down at 8 rpm for 3 min. Then the supernatants were discarded without touching the pellet and pellet was resuspended with 200 μl of Lysozyme by vortexing and was incubated for 1 hour at 37°C . For each sample 20 μl of proteinase K was added and 200 μl of buffer LB_1 also added to the mixture. Mixing was done by vortexing and incubated for 15 min at 60°C (Lysis step). 200 μl of 100 % ethanol was added to the mixture and vortexed. Mixture was applied to the spin column and centrifuged at 8 rpm for 1 min. (Binding step). The liquid flow-through together with the collection tube was discarded and spin column was placed in a new collection tube. 500 μl of buffer W_2 was added to the spin column (Wash step:01). Then centrifused at 8 rpm for 1 min. The liquid flow-through together with the collection tube was discarded and the spin column was placed in a new collection tube. 500 μl of Buffer W_2 was added to the spin column (wash step :02) and centrifused at 8 rpm for 1 min again. The liquid flow-through together with the collection tube was discarded and the spin column was placed in a new collection tube again. The empty spin column was centrifused at 12.5 rpm for 1 min. The liquid flow-through together with the collection tube was discarded under the spin column into medical waste. The spin column was placed in a clean 1.5 ml of microcentrifuse tube and waited for 1 min to evaporate ethanol. 100 μl of Buffer EL_3 was added to the spin column and waited for 5 min (Elusion step), then centrifused at 12.5 rpm for 5 min. At last the liquid flow-through was the DNA sample. The extracted DNA samples were stored at -20°C . PCR Master Mix 01 contains a highly specific and accurate Taq DNA Polymerase (with hot- start property), the PCR buffer and the dNTP Mix. PCR Master Mix 01 also contains forward and reverse primers and dual labelled probes specific for Candida albicans genomes and for the internal control. An internal control was included in the kit to control DNA isolation and PCR inhibition (the internal control is a synthetic DNA molecule). It was added into the biological sample with protinase K during DNA isolation, to control the isolation efficiency and PCR inhibition. 5 μl of internal control was added during isolation per sample. The kit includes synthetic DNA positive control 01(Candida albicans DNA). All the kit components were thawed before used. The reagents composed of PCR Master Mix 01: 20 μl , internal control:0.2 μl , sample DNA (Negative/Positive Control) 5 μl , total volume:25 μl . Those values were multiplied with the sample number to find the values required for the master mix and 5 μl of sample DNA (Negative/Positive Control) was added in PCR tubes. The tube caps were closed and centrifuged.

Programming the Real-Time PCR Instrument:

The thermal protocol for Bosphore Candida Basic Panel Kit v2 was composed of an initial denaturation for activation of Taq DNA polymerase, a two-steps amplification cycle and a terminal hold. The real time data was collected at the second step of the amplification cycle. The thermal protocol that was applied for the reaction was indicated as initial denaturation at 95°C for 14:30 min, denaturation at 97°C for 00:30 min, annealing at 60°C for 01:00 min. Those had the number of amplification cycle was 40. At last of the procedure hold was done at 32°C for 2 min. By the end of the thermal protocol, the Real-Time PCR instrument software calculated the baseline cycles and the threshold automatically.

Ethical consideration: This was an in vivo observational cross-sectional study in which sample size was 50 and specimens were taken from infected root canal. After taking departmental permission, ethical clearance for the study was taken from the Ethical Review Committee of BSMMU (No BSMMU/ 2022/ 3981, 21/ 04/ 2022).

Result

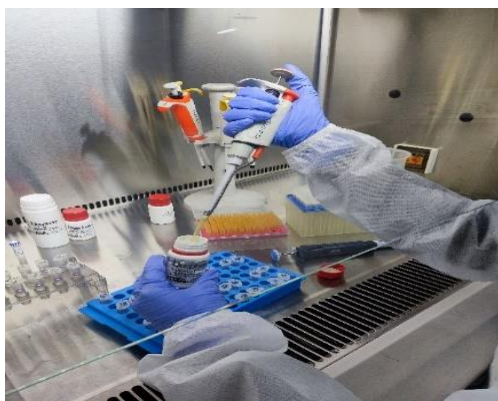
The result of our present study, Real-Time PCR test revealed expected validity to detect the presence of candida albicans than conventional culture test. In our total 50 samples of 50 patients 22 (44 %) samples detected amplification curve positive for candida albicans using Bosphore Candida Basic Panel Kit V2 Anatolia, Gene-works (Manufacturer) Istanbul, Turkey (Table 1). On the other hand, we used selective SDA media (Himedia Laboratories, Mumbai India) for culturing candida albicans. 10 (20%) samples of 50 patients/samples showed positive growth for candida albicans (Table 2). Real-Time PCR showed more than two fold frequency and statistically significant (P value < 0.0001). 22 samples in teeth with pulpal infection and 28 were negative out of total 50 samples, regarding the comparison of culture test result in which only 10 samples showed positive and rest of the 40 samples were negative out of 50 samples (Table 3). However those 10 samples which were positive for candida albicans in culture method, same samples were also revealed positive for candida albicans in real time PCR method. On the other hand 12 samples showed the presence of amplification curve for candida albicans but same number of samples was revealed negative in culture. In our study culture test showed 20 % positivity in growth of candida albicans in selective media SDA associated with the microscopic view of both the gram stain and germ tube test. The analysis of real time PCR method, in where real-time PCR machine showed the presence of amplification curve of candida albicans were 44 % positive. There was a significant difference of P value < 0.0001 found between culture test and real-time PCR test was obtained by Chi-square test. The validity test for real-time PCR revealed that 100 % sensitivity, 70 % specificity, 45.45 % PPV, 100 % NPV and accuracy is 76 %.



Figure 01 (A): Candida growth on SDA.



Figure 01 (B): Microscopic view of Candida albicans.



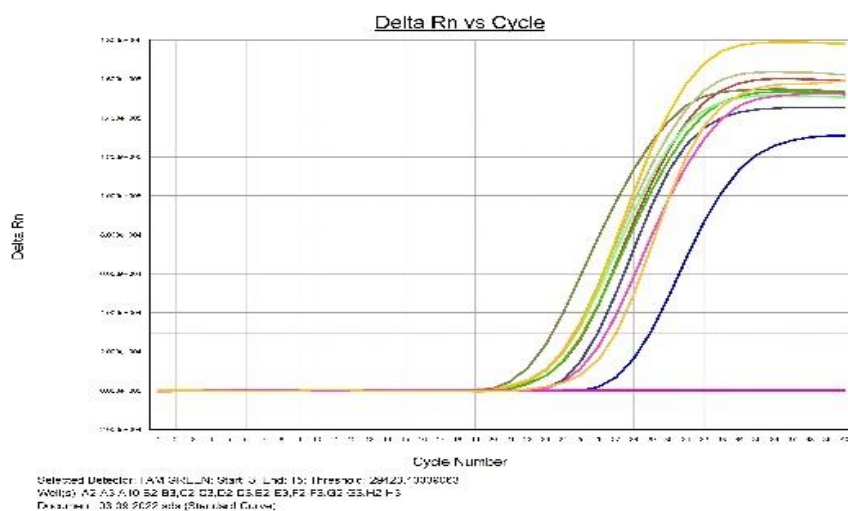


Figure 02: Real-time PCR analysis.

Table-1: Distribution of the study patients by culture test (N=50)

Culture test results	Frequency	Percentage (%)
Positive	10	20.0
Negative	40	80.0
Total	50	100.0

Table-1 showing percentage of candida albicans by culture test: 20 %

Table-2: Distribution of the study patients by PCR test (N=50)

PCR test results	Frequency	Percentage (%)
Positive	22	44.0
Negative	28	56.0
Total	50	100.0

Table-2 showing percentage of candida albicans by PCR test: 44 %

Table-3: Association of PCR and Culture test results in detection of candida albicans in infected root canal (N=50)

		Culture			p-value
		Positive	Negative	Total	
PCR	Positive	10	12	22	<0.0001*
		TP	FP		
	Negative	0	28	28	
		FN	TN		
Total		10	40	50	

p-value obtained by Chi-square test, *significant

Table-4: Diagnostic performance test was done to detection by PCR and Culture of candida albicans in infected root canal (n=50)

Results	Values	95% CI
Sensitivity	100.0%	69.2% to 100.0%
Specificity	67.5%	50.8% to 81.4%
Positive Predictive Value	43.5%	32.9% to 54.6%
Negative Predictive Value	100.0%	-
Accuracy	74.0%	59.7% to 85.4%

Discussion

The findings of this study reveal that out of 50 samples, 22 samples (44%) contained candida albicans by Real-time PCR method and 10 samples (20%) out of 50 were positive for candida albicans. The result of the current study is both comparable to and different from those of some of the earlier investigations. In our investigation, it was discovered that infected root canal samples had a prevalence of candida albicans was 44% by Real-time PCR method. On the other hand 20% was by culture method with significant p value. Higher accuracy of diagnostic performance was found in the present study in real-time PCR in comparison with culture method. There was a significant difference of P value < 0.0001 found between culture test and real-time PCR test was obtained by Chi-square test. The validity test for real-time PCR revealed that 100 % sensitivity, 70 % specificity, 45.45 % PPV, 100 % NPV and accuracy is 76 % according to formula.

In a research by Al-Sakati et al¹⁸, 42/42 plaque samples had bacterial growth, whereas 32/42 gutta-percha samples had bacterial growth with a dominance of *Streptococcus* spp. (12/42) and *Enterococcus faecalis* (9/42). The mean number of bacterial taxa per gutta-percha sample was 1.6 cultivatable taxa, significantly lower than in the plaque sample that had six taxa/sample ($P < 0.001$). Fungus-specific cultures were negative for gutta-percha samples, and only one plaque sample had growth of a fungus. In total, 36/42 plaque samples were positive in bacterial Pan-PCRs. In bacterial Pan-PCRs of 31/42 gutta-percha samples, dominant microorganisms were identified including *Streptococcus* spp. (5/42) and *E. faecalis* (4/42). Moreover, in 7/42 gutta-percha samples, DNA of bacteria which are difficult-to-cultivate in microbiology routine culture. DNA of *Candida* spp. was detected in 5/42 root canals by fungal Pan-PCR (1/5) and genus-specific *Candida*-PCR (5/5).

Similar comparative study conducted by Schabereiter-Gurtner et al.¹⁹ 26 respiratory samples, 4 tissue samples from the maxillary sinus, and 1 blood sample were retrospectively tested and real-time PCR results were compared with results from culture. 20 samples (64.5%) were both culture positive and positive by real-time PCR. 6 (19.4%) showed no growth of fungi but were positive by novel real-time PCR. Result of their study validated 70 % specificity with no change in sensitivity, on the other hand present study validated 70 % specificity and 10 samples out of 50 (20%) were both culture and real-time PCR positive and 12 samples (24 %) had no growth for candida albicans but positive for PCR. The result of validity test in study of Pourhajibagher et al.²⁰ was almost co-related with the validity test of present study in where multiplex real-time PCR could represent the presence of all target microorganisms in 100% cases before and after the Photo-activated Disinfection (PAD) in where validity of multiplex real-time PCR was similar to present study. Before PAD, they used the culture method, *Enterococcus faecalis* (100%) was found to be the most frequent, whereas after PAD these microbial frequencies changed to 80%.

The sensitivity and negative predictive value of the multiplex real-time PCR were 100% before and after the PAD, which was similar with our present study because in this study the sensitivity and NPV (negative predictive value) was also 100 % in detection of candida albicans by using real-time PCR. The highest and the lowest specificities were 100% and 82% before PAD, and 97% and 89% after PAD for *E. faecalis* and *P. gingivalis*, respectively in their study. So they observed from the results, multiplex real-time PCR demonstrated high sensitivity and specificity when compared to the culture technique which was also proved in our study. Therefore, it can prove to be a highly sensitive technique to detect the endodontic infections microflora. Baumgartner et al⁹ evaluated the contents of infected root canals and aspirates of cellulitis/abscesses of endodontic origin for the presence of *Candida albicans* using the conventional PCR. In their study, PCR primers specific for the 18S ribosomal RNA gene of *C. albicans* were used to survey 24 samples taken from infected root canals and 19 aspirates from peri-radicular infections of endodontic origins. The presence of *C. albicans* was detected in 5 of 24 (21%) samples taken from root canals, but none was detected in the peri-radicular aspirates whereas in our study, 22 of 50 (44%) samples revealed positive for candida albicans by analysis with real- time PCR. It had claimed that PCR is an extremely sensitive molecular method that may be used to identify *C. albicans* directly in samples from infections of endodontic origin.

Dumani et al.¹⁷ explored the frequency of two important pathogenic microorganisms associated with endodontic infections, *Enterococcus faecalis* and *Candida albicans*, in root canal samples from patients with necrotic pulps or failed canal therapy was explored using polymerase chain reaction method. In their study, results were dissimilar in the frequency of *Candida albicans* within infected root canal with our study. They collected 117 microbial samples teeth with necrotic pulp tissues and 114 teeth with failed endodontic treatment. Their results revealed that *E. faecalis* were identified in 16% of the necrotic and 10% of the retreated root canal infections by PCR. *Candida albicans* genome was identified in 20% and 11% of the necrotic and retreated root canal infections, respectively, by PCR. The frequencies of microbiota were not statistically different between necrotic and retreatment groups ($p > 0.05$, chi squared test).

That study concluded analysis using PCR, teeth with periapical lesions revealed that *E. faecalis* was found in fewer patients than in previous studies. The *C. albicans* prevalence was consistent with previous reports. No statistical difference was found between primary and secondary root canal infections for *C. albicans* or *E. faecalis*. But in present study 44 % *Candida albicans* positive responsible for infection in root canal using real-time PCR.

While comparing the Pan-PCR method with culture-dependent approach, Al-Sakati et al.¹⁹ identified dominant microorganisms including *Candida albicans* in root filled teeth with apical periodontitis, result revealed statistically significant (significance level was set at $P < 0.05$) was similar with present study. Their study samples were gutta-percha removed from 42 teeth with periapical radiolucencies undergoing root canal retreatments and subgingival plaque. Real-Time Pan-PCRs were conducted for the diagnosis of predominant fungi targeting ITS1-2 region and 1/42 plaque sample had growth of a fungus. DNA of *Candida* spp. was detected in 5/42 root canals by fungal Pan-PCR (1/5) and genus-specific *Candida* PCR (5/5). So, they Concluded that Pan-PCR assays remain appropriate as a broad-range approach for the detection of a dominant pathogen in gutta-percha samples which have less diverse microbial composition. The molecular genetic Pan-PCR approach has the advantage of detecting microorganisms that are as-yet-uncultivable or difficult-to-cultivate and should be therefore complement conventional microbiological diagnostics.

From review of several articles, Mergoni et al.¹¹ excluded from 2225 unique records, 2118 were on the basis of title and abstract. Of the remaining 107 studies, 50 were excluded after full-text review, and 57 were included for qualitative and quantitative analysis. The overall prevalence of *Candida* spp. in root canal infections was 8.20% (95% confidence interval, 5.56%–11.21%). *Candida albicans* was the most frequently isolated species ($p < .001$, $I^2 = 86.07\%$)

A study investigated by Pourhajibagher et al.¹ the microorganisms associated with primary and secondary endodontic infections via culture methods, biochemical tests, and molecular approaches in an Iranian population. Microbial specimens were collected from 36 patients with primary endodontic infection and 14 patients with a history of root canal therapy. Within the total 218 cultivable isolates, frequency of *Enterococcus faecalis* was (36.6%), the most predominant microorganism in secondary endodontic infections and *Candida albicans* was 20% that was similar to present study.

Approximately 96.3% of microbial isolates were identified using biochemical analysis and API® 20A Kit. *Enterococcus faecalis* and *Candida albicans* were the most common microorganisms in the secondary infected root canals with a frequency of 36.6%, 20%.

Conclusion:

It can be concluded, the result of this study revealed that Real-Time Polymerase Chain Reaction method is more sensitive, specific and accurate than that of conventional culture method to identify *Candida albicans* in infected root canal.

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