ASDJ
AINSHAMS DENTAL JOURNAL

Print ISSN 1110-7642
Online ISSN 2735-5039

AIN SHAMS DENTAL JOURNAL
Official Publication of Ain Shams Dental School
March2025 • Vol. 37

Assessment of access cavity disinfection for bacterial DNA and RNA before root canal sampling using molecular analysis

Omar Talib khaleefah[1], Ahmed M. El-Baz[2], Amany Elsaid Badr[1]

Aim: This study aimed to evaluate the bacterial DNA and RNA levels within the access cavity before and after disinfection.
Materials and methods: Twenty-seven patients with necrotic teeth were included. Strict aseptic measures were applied, including plaque removal, disinfection of teeth, and application of rubber dam, using 30% hydrogen peroxide (H2O2) followed by 2.5% sodium hypochlorite (NaOCl). NaOCl activity was neutralised using 10% sodium thiosulfate with saline as the final rinse. After access cavity preparation, bacterial DNA and RNA samples were collected using sterile paper points, before and after using the same disinfection protocol. Samples were verified by Real-time polymerase chain reaction using universal 16S rRNA primers. Data were analysed using the Wilcoxon matched pairs and Fisher's exact tests. The significance level was set at $P<0.05$.
Results: Bacterial DNA and RNA levels were reduced following a disinfection protocol ($P<0.0001$). Bacterial DNA was detected in all the pre- and post-disinfection samples. There was a significant decrease in the proportion of patients with detectable RNA levels after disinfection ($P<0.0001$).
Conclusion: Access cavity disinfection before root canal sampling is crucial to avoid bacterial infiltration into the root canal.

Keywords: disinfection, DNA, endodontic treatment, real-time PCR, RNA

1. Department of Endodontics, Faculty of Dentistry, Mansoura University, Mansoura, Egypt.
2. Department of Microbiology and Immunology, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa, Egypt.
Corresponding author: Omar Talib khaleefah, email: omr_ta@yahoo.com

Introduction

Research on root canal infections faces challenges, including field decontamination, sampling, sample transportation to the laboratory, and subsequent laboratory processing for bacterial analyses.[1] The primary challenge lies in decontamination of the access cavity, which is an essential preparatory step for creating a sterile working field. The access cavity should be separated from extraneous microbes and checked using a contamination control sample to confirm the absence of contaminants before entry into the pulp space.[1,2]

Microorganisms present on the tooth surface or within the access cavity may be inadvertently collected during the sampling process, potentially leading to contamination and affecting the accuracy of the root canal sample results. As our objective was to obtain samples from the root canal, the access cavity was the most relevant site. It is reasonable to anticipate that the cavity walls are devoid of contamination before entering the pulp chamber.[1,2]

An essential first step is to operate in a sterile environment devoid of external microorganisms. Subsequently, the field should be regularly assessed using a contamination-control culture or DNA sample to verify the absence of contaminants before gaining access to the pulp space.[1] Traditional culturing methods detect viable microorganisms that grow and form visible colonies on agar plates or in broth cultures. The colonies are then subjected to phenotypic and biochemical tests for species identification.[1,3] The major drawbacks of cultivation-based approaches include multiple limitations in cultivation and challenges in identifying numerous cultivable species.[4,5]

It is important to recognise that sterility is defined differently for each method. A sterile culture field implies the absence of cultivable viable microbes, whereas, for DNA samples, this signifies the absence of bacterial DNA from the sampled area.[1] In order to target viable bacteria, it is essential to recognise that using DNA alone may not be a highly reliable marker. Non-viable bacteria can retain their DNA. However, RNA is closely associated with the metabolic activity of cells, making it a more precise indicator of bacterial viability.[6]

RNA is an essential molecule involved in gene expression and cellular metabolism. Unlike DNA, RNA is rapidly degraded by dead bacterial cells, making it a reliable marker of viability. Therefore, assessing RNA levels provides a direct insight into the metabolic activity and viability of bacterial populations within the access cavity.[6,7]

To the best of our knowledge, no previous study has evaluated bacterial DNA and RNA within the access cavity prior to obtaining root canal samples. This study aimed to evaluate the disinfection protocol used to sterilise access cavities from microbial residues by detecting bacterial DNA and RNA using real-time polymerase chain reaction (PCR) as a sensitive molecular technique.

Materials & Methods

Patient selection

This study was approved by the Research Ethics Committee of the Faculty of Dentistry, Mansoura University (approval number A01050422) and registered on clinicaltrials.gov under the identifier NCT06291623.

Twenty-seven patients who required endodontic treatment were selected from the outpatient clinic of the Department of Endodontics, Faculty of Dentistry, Mansoura University. Patients meeting the following criteria were included in the study: single-rooted teeth with necrotic pulp, tooth unresponsive to sensitivity testing, and patient age between 19 to 60 years. All

selected participants received detailed information about the procedures and were requested to provide written informed consent following the ethical guidelines of the Faculty of Dentistry, Mansoura University.

Patients presenting with any of the following conditions were excluded from the study: recent antibiotic use within the past 3 months, presence of systemic diseases, inability to isolate the tooth with a rubber dam, periodontal pockets exceeding 3 mm in depth, and teeth with root fracture or extensive decay.

Procedure

For each patient, the following procedural steps were performed: after isolating the teeth with a rubber dam, strict aseptic protocols were applied, including plaque removal, application of a rubber dam, and disinfection of teeth using 30% hydrogen peroxide (H_2O_2) followed by 2.5% sodium hypochlorite (NaOCl). The NaOCl was neutralised with 10% sodium thiosulfate. The teeth were anaesthetised, followed by access cavity preparation using sterile diamond burs and flushed with sterile saline to thoroughly eliminate the carious lesion and old restoration.

Following preparation and saline flushing of the access cavity, three sterile paper points were immediately used to obtain a sample (S1) from the access walls. The access cavity was disinfected as outlined in the aforementioned protocol and a second sample (S2) was collected. After sampling, all patients underwent endodontic treatment, which included the use of flexible files, irrigation, obturation, and subsequent final restoration.[8]

Molecular analysis

The microbiological sampling methods have been outlined previously.[9,10]. All specimens were transferred to cryotubes filled with RNAlater solution (Qiagen, Hilden, Germany) and promptly placed in an icebox before being stored at -80°C in the laboratory for future DNA and RNA extraction. Total nucleic acid was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicenter Technologies, Madison, WI, USA) in accordance with the manufacturer's instructions, as detailed in previous reports.(4) After the extraction process, the total nucleic acid samples were suspended in 35 µL of Tris-EDTA buffer (Epicentre Technologies) to maintain pH stability. Subsequently, the samples were divided into two vials: one was stored at -20°C for subsequent DNA analysis, while the other underwent RNA purification.

DNase treatment was conducted to ensure the absence of contaminating DNA in the RNA samples, which was confirmed by PCR using universal primers (16S rRNA) designed specifically for oral bacteria.[12] RNA and DNA concentrations were assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA samples were converted to cDNA using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis kit (Epicentre Technologies, Madison, WI, USA), following the manufacturer's instructions. Blinded assessment was implemented during the molecular analysis, sample identities were anonymized to ensure that the analyst did not know which sample belonged to which subject or phase. The resulting cDNA was subjected to real-time PCR for quantitative analysis.

The mean values of DNA and cDNA measurements were used to calculate the number of 16S rDNA and 16S rRNA copies per access cavity sample. DNA data were used for bacterial quantification, whereas RNA (cDNA) data were used to estimate bacterial activity in samples positive for DNA. DNA and cDNA from all samples

were evaluated to assess the viable bacterial load in the access cavity before and after the treatment.

Standard curve

A standard curve was created using the clinical strain *Enterococcus faecalis* to generate a linear regression curve between log concentration and Ct to detect unknown DNA concentrations, as mentioned in previous studies.[13-15]

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.0; GraphPad Software, San Diego, CA, USA). Numerical variables are presented as mean ± standard deviation (SD). Data were tested for the normality of distribution using the Shapiro–Wilk test. The difference between pre- and post-treatment was tested using the Wilcoxon matched-pair test. The effect of disinfection was tested in association with the presence or absence of RNA using the Fisher's exact test. Statistical significance was set at $P<0.05$.

Results

The participants included 13 women aged between 25 and 39 years and seven men aged between 27 and 41 years. A standard curve was constructed (R^2 = 0.9898) by qPCR amplification of pure genomic *E. faecalis* DNA. The resulting standard curve validated the linear correlation between the exponential growth in DNA concentration and Ct values (Table 1, Figure 1).

Bacterial DNA was detected in all the pre- and post-disinfection samples. Pre-disinfection samples from all patients showed the presence of RNA, whereas after disinfection, only nine samples had detectable RNA. Additionally, there was a substantial decrease in the proportion of patients exhibiting detectable RNA levels after disinfection ($P<0.0001$, Fisher exact test). The Wilcoxon matched-pairs test demonstrated a statistically significant reduction in post-disinfection DNA and RNA levels ($P<0.0001$) (Figures 2 and 3).

Table 1: cDNA results for all participants in the study

cDNA expression			
Patient		Pre-treatment	Post-Treatment
1	**F**	+	-
2	**F**	+	+
3	**F**	+	+
4	**F**	+	+
5	**M**	+	+
6	**F**	+	-
7	**M**	+	+
8	**F**	+	-
9	**F**	+	+
10	**F**	+	+
11	**F**	+	-
12	**M**	+	-
13	**F**	+	-
14	**F**	+	-
15	**M**	+	-
16	**F**	+	-
17	**F**	+	-
18	**M**	+	-
19	**F**	+	-
20	**M**	+	-
21	**F**	+	-
22	**F**	+	+
23	**F**	+	-
24	**M**	+	-
25	**M**	+	-
26	**F**	+	-
27	**F**	+	+
No. of cDNA +ve		**27 (100%)**	**9 (33.33%)**
No. of cDNA -ve		**0 (0%)**	**18 (66.67%)**
Significance (Fisher's exact test)		***P* value <0.0001**	

F, female participant; M, male participant

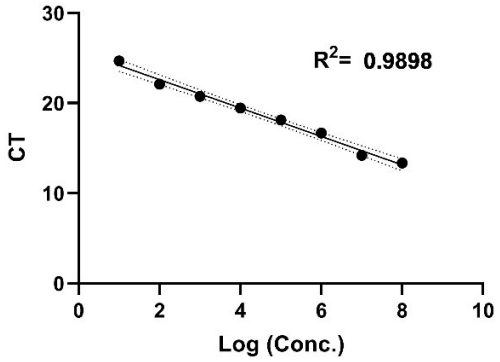

Figure 1: A standard curve specific to Enterococcus faecalis OS16.

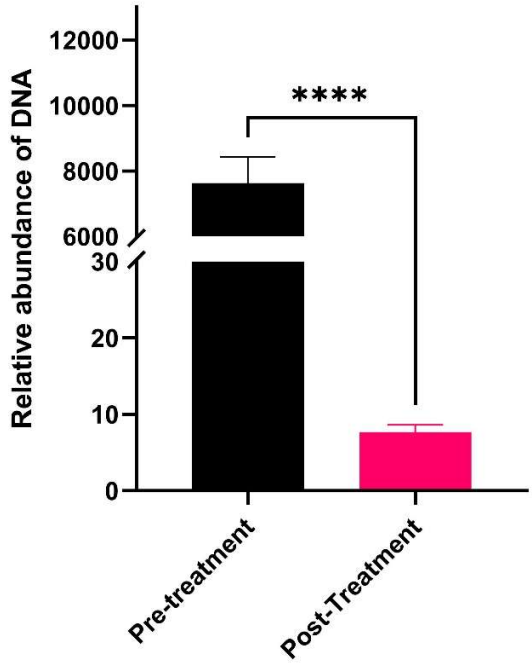

Figure 2: Effect of treatment on DNA relative abundance.Data are presented as mean ± SD. Values were compared using Wilcoxon matched-pair signed rank test; ****P< 0.0001.

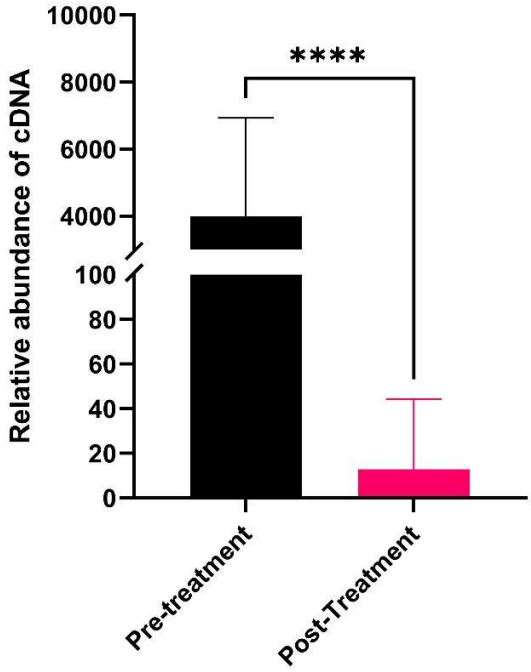

Figure 3: Effect of treatment on cDNA relative abundance. Data are presented as mean ± SD. Values were compared using Wilcoxon matched-pair signed rank test; ****P< 0.0001

Discussion

Despite the implementation of the disinfection protocol, the study revealed that the complete elimination of bacterial DNA and RNA from the tooth access cavity was not achieved. Real-time PCR analysis utilising universal 16S rRNA gene primers specifically designed for oral bacterial detection was used to identify bacterial DNA in all access cavity samples. Although there was a noticeable reduction in the number of DNA-contaminated samples after decontamination, complete eradication was not achieved.

When assessing the microbial content of the root canal before and after treatment, it is essential to remove bacteria from the tooth surface and access the cavity prior to accessing the pulp to maintain the integrity and validity of microbial analyses in endodontic research and clinical practice.

Neglecting this step could result in the contamination of the root canal samples, leading to inaccurate findings.[1]

In endodontic treatment, only viable bacteria affect treatment outcomes, emphasising the importance of targeting and eliminating active microbial populations to ensure successful treatment.[16] Culturability and metabolic activity are the commonly recognised criteria for assessing bacterial viability.[17]

Bacterial culturability can be measured by determining the ability to produce colonies. However, bacteria may enter a viable but non-culturable (VBNC) state owing to unfavourable conditions, and in such scenarios, they cannot be evaluated based on culturability criteria.[18] RNA analysis is an alternative approach for detecting VBNC bacteria by evaluating their metabolic activity. This method involves the assessment of RNA expression, which reflects the ongoing metabolic processes within bacterial cells. Unlike traditional culturability tests, which may fail to detect VBNC bacteria, RNA analysis provides insights into the actual metabolic state of bacteria, allowing for a more accurate assessment of their viability.[18,19]

This study is the first attempt to evaluate bacterial RNA within the access cavity. This approach is particularly significant because it targets viable bacteria, which is crucial for understanding microbial activity and potential treatment outcomes. We focused on bacterial RNA as an indicator of active metabolic processes. The main aim of analysing access cavity control samples was to evaluate the efficiency of the disinfection procedure in eradicating viable bacteria.

We used a standard curve to quantitatively assess the DNA samples by comparing their signal response to that of the standard curve.[14] *E. faecalis* was selected as the standard organism for constructing the standard curve because of its prevalence in the oral cavity of endodontic patients, as evidenced by studies that utilised both culture-based and molecular detection methods.[20-26]

Molecular techniques such as real-time PCR, which targets bacterial DNA and RNA, enable researchers to detect and quantify viable bacterial populations. In this study, real-time PCR was employed as the quantification method because of its high sensitivity. It can detect and quantify not only cultivable bacteria but also culture-difficult and non-cultivable species.[27-29]

Our findings on the presence of bacterial DNA within the access cavity align closely with those of previous studies.[30,31] Although no previous studies have assessed bacterial RNA within the access cavity, employing RNA in this study facilitated the assessment of bacterial activity levels within the access cavity. Notably, the results suggest that bacteria can sustain metabolic activity even after disinfection.

The persistence of residual bacterial genetic material indicated potential limitations in the effectiveness of the protocol employed. These findings highlight the complexity and challenges involved in achieving complete sterilisation of the access cavity of the tooth. Despite efforts to disinfect the cavity thoroughly, traces of bacterial genetic material remained, underscoring the need for further disinfection optimisation.

The presence of residual bacterial DNA and RNA highlights the need for continued research and refinement of disinfection protocols to enhance efficacy and ensure optimal patient outcomes. Furthermore, these results emphasise the importance of employing sensitive molecular techniques for the thorough evaluation of disinfection procedures, as they provide valuable insights into the effectiveness of treatment interventions beyond traditional microbiological assays.[27,32,33]

The limitation of this study was its sole focus on assessing viable bacteria within the access cavity, overlooking the examination of bacterial presence on the tooth surface and rubber dam, owing to cost constraints associated with molecular analyses. Although valuable insights into treatment efficacy were gained, overlooking other potential sources of contamination may limit the comprehensiveness of the findings. Future research with ample resources should aim to investigate viable bacteria across all relevant surfaces within the endodontic environment for a more comprehensive understanding of microbial colonisation and treatment outcomes.

Conclusion

Access cavity disinfection before root canal sampling is crucial to prevent bacterial infiltration. The presence of residual bacterial DNA and RNA highlights the need for refining disinfection protocols to improve efficacy and patient outcomes. Further research is necessary to optimize these protocols and enhance their effectiveness.

Funding

This research was entirely self-funded by author(s). No external financial support was received.

Data availability

This study is part of a PhD thesis in Mansoura University. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations of Ethics approval and consent to participate

This study was approved by the Research Ethics Committee of the Faculty of Dentistry, Mansoura University (approval number A01050422). All selected participants were requested to provide written informed consent in accordance with the ethical guidelines of the Faculty of Dentistry, Mansoura University.

Competing Interests

The authors declare no conflicts of interest.

References

1. Figdor D, Brundin M. Contamination Controls for Analysis of Root Canal Samples by Molecular Methods: An Overlooked and Unsolved Problem. *J Endod*. 2016;42(7):1003-8.
2. Möller AJ. Microbiological examination of root canals and periapical tissues of human teeth. Methodological studies. *Odontol Tidskr*. 1966;74(5):1-380.
3. Vianna ME, Horz HP, Gomes BP, Conrads G. Microarrays complement culture methods for identification of bacteria in endodontic infections. *Oral Microbiol Immunol*. 2005;20(4):253-8.
4. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 1--current molecular technologies for microbiological diagnosis. *J Endod*. 2005;31(6):411-23.
5. Bursle E, Robson J. Non-culture methods for detecting infection. *Aust Prescr*. 2016;39(5):171-5.
6. Kim SY, Shin Y, Lee CY, Jung IY. In vivo quantitative evaluation of live and dead bacteria in root canal infection by using propidium monoazide with real-time PCR. *J Endod*. 2013;39(11):1359-63.
7. Chełkowska-Pauszek A, Kosiński JG, Marciniak K, Wysocka M, Bąkowska-Żywicka K, Żywicki M. The Role of RNA Secondary Structure in Regulation of Gene Expression in Bacteria. *Int J Mol Sci*. 2021;22(15):7845.
8. Khaleefah O, El-Souda AA, Badr AE-S. Cyclic fatigue and fracture surfaces evaluation of different nickel titanium endodontic files. *Egypt Dent J*. 2021;67(1-January (Conservative Dentistry and Endodontics)):883-91.
9. Nakamura VC, Pinheiro ET, Prado LC, et al. Effect of ultrasonic activation on the reduction of bacteria and endotoxins in root canals: a randomized clinical trial. *Int Endod J*. 2018;51 Suppl 1:e12-2.
10. Carvalho APL, Nardello LCL, Fernandes FS, et al. Effects of Contemporary Irrigant Activation Schemes and Subsequent Placement of an Interim Dressing on Bacterial Presence and Activity in

Root Canals Associated with Asymptomatic Apical Periodontitis. *J Clin Med*. 2020;9(3):854.
11. Nardello LCL, Vilela BG, Fernandes FS, et al. Analysis of Active Bacteria Persisting after Chemomechanical Procedures: An RNA- and DNA-based Molecular Study. *J Endod*. 2020;46(11):1570-6.
12. El-Baz AM, Khodir AE, Adel El-Sokkary MM, Shata A. The protective effect of Lactobacillus versus 5-aminosalicylic acid in ulcerative colitis model by modulation of gut microbiota and Nrf2/Ho-1 pathway. *Life Sci*. 2020;256:117927.
13. Al-Zubidi M, Widziolek M, Court EK, et al. Identification of Novel Bacteriophages with Therapeutic Potential That Target *Enterococcus faecalis*. *Infect Immun*. 2019;87(11):e00512-19.
14. Rôças IN, Lima KC, Siqueira JF Jr. Reduction in bacterial counts in infected root canals after rotary or hand nickel-titanium instrumentation--a clinical study. *Int Endod J*. 2013;46(7):681-7.
15. Almelan MF, Abbas UK, Al-Zubidi M. Isolation and characterization of bacteriophage targeting Enterococcus faecalis isolated from root canal infection (in vitro study). *Biomedicine*. 2023;43(3):977-82.
16. Siqueira JF Jr, Rôças IN. Diversity of endodontic microbiota revisited. *J Dent Res*. 2009;88(11):969-81.
17. Nocker A, Camper AK. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. *FEMS Microbiol Lett*. 2009;291(2):137-42.
18. Trinh KTL, Lee NY. Recent Methods for the Viability Assessment of Bacterial Pathogens: Advances, Challenges, and Future Perspectives. *Pathogens*. 2022;11(9):1057.
19. Guo L, Ye C, Cui L, et al. Population and single cell metabolic activity of UV-induced VBNC bacteria determined by CTC-FCM and D2O-labeled Raman spectroscopy. *Environ Int*. 2019;130:104883.
20. Gomaa MA, Elhawary YM, Badr AE. Glycyrrhizin Enhances the Proliferation of Diabetic Bone Marrow-derived Mesenchymal Stem Cells: A Potential Therapeutic Agent in Endodontic Surgery. *J Contemp Dent Pract*. 2023;24(7):494-9.
21. Molander A, Reit C, Dahlén G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. *Int Endod J*. 1998;31(1):1-7.
22. Gaeta C, Marruganti C, Ali IAA, et al. The presence of *Enterococcus faecalis* in saliva as a risk factor for endodontic infection. *Front Cell Infect Microbiol*. 2023;13:1061645.
23. Siqueira JF Jr, Rôças IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2004;97(1):85-94.
24. Wang QQ, Zhang CF, Chu CH, Zhu XF. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. *Int J Oral Sci*. 2012;4(1):19-23.
25. Ashry SH El, Nagy MM, Elsewify TMA, Allah Ali EBEDA. Efficiency of Newly Introduced Root Canal Irrigants Based on Nano Particles (In Vitro Study). Ain Shams Dent J. 2022;27(3):44–55.
26. Elbanna A, Elmesellawy MY, El-Refai DA. Antibacterial behavior of calcium fluoride-modified tricalcium silicate cement against different oral bacterial strains. Ain Shams Dent J. 2024;34(2):76–87.
27. Cook J, Nandakumar R, Fouad AF. Molecular- and culture-based comparison of the effects of antimicrobial agents on bacterial survival in infected dentinal tubules. *J Endod*. 2007;33(6):690-2.
28. Rôças IN, Siqueira JF Jr. In vivo antimicrobial effects of endodontic treatment procedures as assessed by molecular microbiologic techniques. *J Endod*. 2011;37(3):304-10.
29. Rabea DH, Allam GG, El-Aziz AMA, El-Hady SAER. Comparing The Antibacterial Effect of Psidium guajava Extract, Camellia sinensis Extract and Chlorhexidine Gluconate as Root Canal Irrigants in Primary Teeth: In-Vitro Study. Ain Shams Dent J. 2020;19(3):79–86.
30. Ng YL, Spratt D, Sriskantharajah S, Gulabivala K. Evaluation of protocols for field decontamination before bacterial sampling of root canals for contemporary microbiology techniques. *J Endod*. 2003;29(5):317-20.
31. Sedgley C, Buck G, Appelbe O. Prevalence of Enterococcus faecalis at multiple oral sites in endodontic patients using culture and PCR. *J Endod*. 2006;32(2):104-9.
32. Alves FR, Rôças IN, Almeida BM, Neves MA, Zoffoli J, Siqueira JF Jr. Quantitative molecular and culture analyses of bacterial elimination in oval-shaped root canals by a single-file instrumentation technique. *Int Endod J*. 2012;45(9):871-7.
33. Rôças IN, Siqueira JF Jr. Identification of bacteria enduring endodontic treatment procedures by a combined reverse transcriptase-polymerase chain reaction and reverse-capture checkerboard approach. *J Endod*. 2010;36(1):45-52.