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## Research Article

# Herpesviral and “Red Complex” Bacterial Analysis of Acute Apical Abscesses

### Abstract

**Introduction:** To investigate the possible association between presence of Epstein–Barr virus, human cytomegalovirus and three endodontic bacterial pathogens (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*) “red complex” in samples from patients with acute apical abscesses using real-time PCR.

**Methods:** Periapical samples were collected from 18 acute apical abscess specimens and 6 control samples. The apical area of each study tooth was examined in periapical radiographs obtained using a long cone paralleling technique. The study included only single-rooted teeth that had carious lesions, necrotic pulps, and radiographic evidence of periradicular bone loss. No apparent communication from the abscess to the oral cavity or skin surface was observed. DNA extracts from purulent exudate aspirates of acute apical abscess and six control samples were evaluated for the presence of microbial and viral loads using real-time polymerase chain reaction (PCR) following the kit protocols recommended by the manufacturers. The chi squared test was used for statistical analysis.

**Results:** HCMV, EBV, *T. denticola*, *P. gingivalis*, and *T. forsythia* were detected in 16.7 %, 5.6 %, 44.4 %, 27.7 %, and 22.2 % of the samples, respectively. As for the healthy pulps used as noninflamed controls, no control specimens contained bacterial or viral DNA. The pair HCMV/ *T. denticola* and HCMV/ *T. forsythia* was detected in one case. The association between HCMV, *T. denticola* and *T. forsythia* was not statistically significant.

**Conclusion:** Within the limitations of the present study, our PCR based findings revealed that in “red complex” bacteria, *T. denticola* and *T. forsythia* tended to occur in co-infection with HCMV.

## Introduction

An acute apical abscess is an inflammatory disease of endodontic origin caused by a mixed bacterial infection, dominated by anaerobic species and characterised by rapid onset, spontaneous pain, tenderness of the tooth to pressure, purulent fluid formation, and associated swelling [1,2]. The microbial diversity in different forms of periapical pathology, including acute apical abscesses, has traditionally been assessed using anaerobic cultures and culture-independent molecular methods. The molecular methods used most often for microbial identification are the polymerase chain reaction (PCR) method and its variations [3,4].

Using molecular methods, Socransky et al. [5], using DNA-DNA hybridization method, identified a consortium of the bacterial species *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, as having the strongest association with periodontal disease severity. They named this microbial consortium the “red complex” [6]. Slots et al. [7], hypothesised that some types of aggressive periapical pathosis develop as a result of a series of interactions among herpesviruses, bacteria, and host immune reactions, essentially the same mechanism as in marginal periodontitis [2,8]. More recently, Ferreira et al. [2], suggested that the same mechanism might be applicable to apical abscesses of endodontic origin [9].

Eight human herpesvirus species with distinct biological and

clinical characteristics have been described: herpes simplex viruses 1 and 2, varicella–zoster virus, Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), and human herpesviruses 6, 7, and 8 [8,10,11]. The initial herpesvirus infection is followed by a latent phase in host cells, which ensures the survival of the viral genome throughout the lifetime of infected individuals. The  $\alpha$  herpesviruses (herpes simplex and varicella-zoster viruses) establish latency in long-lived nondividing neuronal cells in sensory ganglia. Beta herpesviruses include cytomegalovirus and herpesviruses 6 and 7, which establish latency in bone marrow–derived myeloid progenitor cells. The  $\gamma$  herpesviruses Epstein-Barr virus and herpesvirus 8 are latent in B lymphocytes. Herpesvirus reactivation can occur spontaneously or as a result of concurrent infection, fever, drugs, tissue trauma, emotional stress, and other factors that impair the host immune defences [7]. EBV causes infectious mononucleosis and almost certainly plays a role in the aetiology of nasopharyngeal carcinoma, Burkitt’s lymphoma, and lymphoproliferative disorders in the presence of immunosuppression [7,12]. This virus is usually transmitted by oral secretions or blood [12]. EBV infects relatively long-lived B lymphocytes during both primary and latent infections and can infect the oropharyngeal epithelium [8,9]. HCMV infection is important clinically in pregnant women, immunosuppressed transplant patients, and individuals with acquired immunodeficiency syndrome (AIDS) and is found in the blood and many body secretions, including saliva, urine, semen, and breast milk [11,12]. HCMV infects

many different epithelial cells, endothelial cells, smooth muscle cells, mesenchymal cells, hepatocytes, granulocytes, and monocyte-derived macrophages and resides in the bone marrow myeloid progenitor cells during latency [1,9,12].

Studies have shown that the genotype distribution and seroprevalence of EBV and HCMV differ among populations [8,13]. Additionally, molecular studies have directly compared the endodontic microbiota of patients from different geographic locations and found significant differences in the prevalence of some important species [3,14]. Therefore, it is reasonable to postulate that it is important to determine the presence of EBV, HCMV, and red complex bacteria (*P. gingivalis*, *T. forsythia*, and *T. denticola*) in periapical lesions in different populations. Based on the applicability of molecular techniques for bacteria and virus identification, the hypothesis for this current study is that herpesviruses and red complex bacteria associations play major roles in the pathogenesis of acute apical abscesses. Using purulent exudate specimens collected from patients, real-time PCR was conducted to investigate occurrence of EBV, HCMV, and the three endodontic bacterial pathogens (*P. gingivalis*, *T. forsythia*, and *T. denticola*) in acute apical abscess of endodontic origin.

## Materials and Methods

### Patient selection

Samples were taken from adult patients seeking emergency treatment in the Department of Endodontics, Dental School of Atatürk University [Erzurum, Turkey]. The Ethical Committee in Research of the Dental School of Atatürk University approved the study protocol, and informed consent was obtained from each of the patients. The patient information was coded anonymously.

For each patient, we recorded the age, gender, and clinical symptoms and signs, including pain on occlusion, tenderness to percussion or palpation, swelling, the presence of periapical radiolucency, history of previous dental treatments, probing depth of any periodontal pockets, and the history of previous and present antibiotic therapy. The apical area of each study tooth was examined in periapical radiographs obtained using a long cone paralleling technique. The study included only single-rooted teeth (13 incisors, five canines) from 18 adult patients (13 males, five females) that had carious lesions, necrotic pulps, and radiographic evidence of periradicular bone loss (Table 1). No apparent communication from the abscess to the oral cavity or skin surface was observed.

The diagnostic terminology was based on the guidelines of the American Association of Endodontists consensus conference recommended diagnostic terminology [16]. The diagnosis of an acute apical abscess was based on the presence of spontaneous pain, exacerbated by mastication, and localised swelling, along with fever, lymphadenopathy, or malaise [2]. The inclusion criteria for patients were as follows: individuals in good health (American Society of

Anaesthesia I or II) with no severe systemic diseases (such as bleeding disorders, diabetes mellitus, etc.) and who required tooth extraction or endodontic treatment because of the presence of endodontic disease [17]. The patients' ages ranged from 22 to 36 years. None of the individuals reported being human immunodeficiency virus (HIV) positive. The exclusion criteria were periodontal involvement of the teeth [probing depth >4 mm, with periodontal bone loss], vertical root fracture, or immature teeth with open apices. Immunocompromised patients or patients who were receiving antiviral or immunosuppressive therapy or had received antibiotics up to 3 months before the start of the study were also excluded.

### Study design

Samples were collected using strict asepsis, as described previously [2,9,18]. Abscesses were sampled by aspiration of the purulent exudate from the swollen mucosa over each abscess. After disinfecting the overlying mucosa with 2% chlorhexidine solution, a sterile disposable syringe was used to aspirate the purulent fluid, which was injected immediately into 2-mL CryoTubes containing 0.7 mL of 5% dimethylsulphoxide (DMSO) in tripticase soya broth (TSB). The samples were frozen immediately at  $-20^{\circ}\text{C}$  until they were processed. Healthy dental pulp removed from six upper premolars with no tissue inflammation, no signs of caries, restoration or cracking, extracted for orthodontic reasons was used as a non-inflamed control [2,9]. DNA was extracted from the clinical samples using the QIAamp<sup>®</sup> DNA Mini Kit (Qiagen, Hilden, Germany), following the protocol recommended by the manufacturer.

### DNA extraction

The frozen samples were left at room temperature to thaw and to adjust to room temperature. The samples were vortexed and centrifuged at 14 000 g for 5 min, and then, the supernatants were removed the pellets were used for DNA extraction. The precipitates were resuspended in 200  $\mu\text{L}$  of distilled water. For each sample, a new 1.5-mL micro centrifuge tube was prepared, and 25  $\mu\text{L}$  of Qiagen Proteinase K was pipetted into each one, and then, 200  $\mu\text{L}$  of sample was added to each tube. To mix the proteinase K and samples, mixture was pipetted at least five times. Two hundred microliters of buffer AL was added to the 1.5-mL micro centrifuge tube with the proteinase K and sample mixture. To ensure efficient lysis, proteinase K, the sample and buffer AL are mixed thoroughly to yield a homogeneous solution. This homogenous solution was incubated at  $56^{\circ}\text{C}$  for at least 15 min in a heating block. To precipitate DNA, 250  $\mu\text{L}$  of 100% ethanol (molecular grade) was added to the lysate and vortexed for 20 s and left to incubation for 5 min at room temperature  $20 (\pm 2^{\circ}\text{C})$  (room temperature). The tube containing lysate was centrifuged briefly to remove drops from inside of the lid before tube opening. To collect the DNA, all contents of the lysate in the tube were transferred to QIAamp MinElute column and centrifuged at  $6,000\times g$  for 1 minute. At this stage, the DNA bound to the silica of the QIAamp MinElute column tube. The DNA bound to the silica was washed twice with washing buffer and then dried at ambient temperature. Finally, the DNA was collected in a 1.5-mL microcentrifuge tube with 60  $\mu\text{L}$  of previously heated ( $50^{\circ}\text{C}$ ) elution buffer and centrifuged at  $14,000\times g$ .

**Table 1:** Clinical features of patients.

	M	F	P	S	F	L	M
Apical abscess ( $n=18$ )	13	5	18	18	2	2	15

M male, F female, P pain, S swelling, F fever, L lymphadenopathy, M malaise

## PCR amplification

**Virus DNA Amplification:** To amplify the EBV and HCMV DNAs, a real-time PCR amplification method was used with commercial Fluorion® EBV QNP 1.0 and Fluorion® CMV 3.0 kits (Iontek, Istanbul, Turkey), respectively. The ICycler IQ Multicolour Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used to amplify and detect the DNA of target viruses with virus-specific kits following the manufacturer’s recommendations, and all amplifications were repeated twice to confirm the results for EBV and HCMV.

**Bacterial DNA Amplifications:** Universal primers directed to 16S rDNA (forward/reverse) (5’ TTAAACTCAAAGGAATTGACGG 3’ / 5’ CTCACGACACGAGCTGACGAC 3’) and species-specific primers for *T. denticola* (5’ AGAGCAAGCTCTCCCTTACCGT 3’ / 5’ TAAGGGCGGCTTCAAATAATGA 3’), *P. gingivalis* (5’ TACCCATCGTCGCCTTGGT 3’ / 5’ CGGACTAAAACCGCATACTTG 3’), and *T. forsythia* (5’ ATCCTGGCTCAGGATGAACG 3’ / 5’ TACGCATACCCATCCGCAA 3’) were chosen based on our previous study [18]. The amplification and detection of DNA with the species-specific and universal primers using real-time PCR were performed with the ICycler IQ Multicolour Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For each real-time PCR, IQ SYBR Green SuperMix (Bio-Rad Laboratories) supplemented with 4.5% DMSO was used. A 50-µL total PCR amplification volume for each reaction was placed in each well of a 96-well MicroAmp Optical Reaction Plate and covered with Optical-Quality Sealing Tape (Bio-Rad Laboratories). The DNA amplification conditions for PCR with the universal and species-specific primers for *P. gingivalis* and *T. denticola* were 15 min initial denaturation at 95°C, followed by 50 cycles of 95°C for 30 s, 65°C for 50 s, and 72°C for 45 s, with a final 45 s at 75°C. The amplification conditions for *T. forsythia* were the same, except that annealing was performed at 60°C for 50 s. The PCR amplifications with species-specific and universal primers were repeated twice to confirm the results. Positive results were obtained in the melting analysis of each amplicon based on the target region based on the primers specific for each bacterium investigated. From among the positive results, one positive amplicon for each bacterium was selected randomly for sequencing by Iontek (Istanbul, Turkey) using an ABI Prism 310 Genetic Analyzer to verify the positive results.

## Statistical analysis

The prevalence of EBV, HCMV, *P. gingivalis*, *T. forsythia*, and *T. denticola* DNA in acute apical abscess lesions was recorded as a percentage of the cases examined. The chi-squared test with Yates’s correction or the Fisher’s exact test was used to analyse the significance of differences. The latter was used whenever at least one cell of the 2 × 2 contingency table had a value <5. Significance levels were established at 5% (< 0.05).

## Results

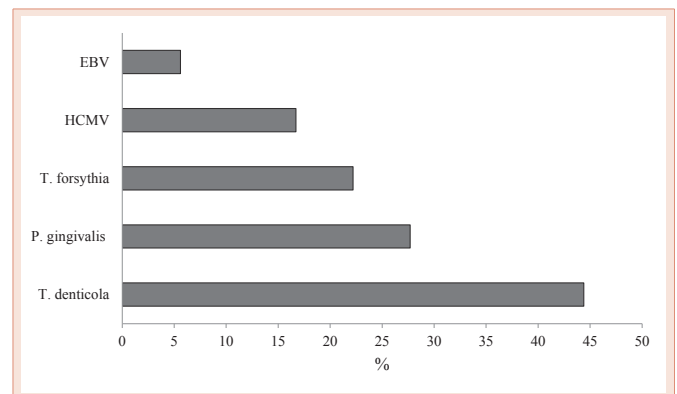
Eighteen apical abscess specimens were collected from adult patients (average age 29 [range 22–36] years). All of the samples

taken from apical abscesses and healthy pulp were positive in the PCR assay targeting the beta-globulin gene. This indicated that the DNA extraction and multiple displacement amplification protocols were effective at making DNA available for detecting viruses and bacteria [18].

Of the 18 abscess samples positive for the beta-globin gene, four (22%) were positive for at least one of the target human viruses. Figure 1 summarises the prevalence of EBV and HCMV DNA in the apical abscesses. HCMV DNA was found in three samples (16.7%), and EBV DNA was found in one (5.6%). Viral coinfection was not observed in any of the 18 apical abscesses studied.

Figure 1 also illustrates the incidence of “red complex” species in the acute apical abscesses. At least one member of the “red complex” was found in 66.6% (12/18) of the cases. *T. denticola*, *P. gingivalis*, and *T. forsythia* were detected in 44.4, 27.7, and 22.2% of the samples, respectively. Five samples (27.7%) yielded two members of the red complex: the pair *P. gingivalis*/*T. denticola* was detected in two cases, *P. gingivalis*/*T. forsythia* in one case, and *T. denticola*/*T. forsythia* in two cases. The complete “red complex”, composed of *P. gingivalis*, *T. denticola*, and *T. forsythia*, was not detected in any samples taken from acute apical abscesses.

Viral-bacterial coinfection was observed in two samples (2/18 cases, 11%) the pairs HCMV/*T. denticola* and HCMV/*T. forsythia* were detected in one case each. The association between HCMV, *T. denticola* and *T. forsythia* was not statistically significant. ( $p > 0.05$ ). EBV was not associated with any of the red complex bacteria in the acute apical abscess samples (Table 2).



**Figure 1:** Prevalence of members of red complex and HCMV, EBV DNA in 18 samples from apical abscess lesions.

**Table 2:** Distribution of bacterial and viral associations in samples from apical abscess lesions.

Bacterial and viral types	<i>P. gingivalis</i> / <i>T. denticola</i> n (%)	<i>P. gingivalis</i> / <i>T. forsythia</i> n (%)	<i>T. denticola</i> / <i>T. forsythia</i> n (%)	HCMV/ <i>T. denticola</i> n (%)	HCMV/ <i>T. forsythia</i> n (%)
Positive acute apical abscess specimens	2 (11.1%)	1 (5.5%)	2 (11.1%)	1 (5.5%)	1 (5.5%)

## Discussion

The purpose of this study was to investigate the possible associations of EBV, HCMV, and three candidate endodontic bacterial pathogens (*P. gingivalis*, *T. forsythia*, and *T. denticola*) in acute apical abscesses using real-time PCR. The result of this study rejects the null hypothesis and has shown that EBV, HCMV and red complex bacteria associations in abscess lesions were not significant.

Concomitant infection with herpesviruses and specific bacterial species has been suggested as applicable to the aetiology of abscesses [15,18]. Sabeti and Slots [15], reported that most anaerobic bacteria (*P. gingivalis*/*P. endodontalis*) were isolated from periapical lesions showing HCMV and EBV dual infection where the lesions were symptomatic or large. Ferreira et al. [18], demonstrated that bacterial and viral DNA occurred concomitantly in two-thirds of acute apical abscess samples.

Molecular methods have been used widely to identify microorganisms in samples without the need for culture. In this study, using real-time PCR, *P. gingivalis*, *T. denticola*, and *T. forsythia* were detected in 27.7, 44.4, 22.2 %, respectively, of the acute apical abscess samples. As with any method, molecular methods have their advantages and limitations. The real-time fluorescence-based PCR method used here is a valuable method that is rapid, identifies PCR products directly without the use of agarose gels, and limits contamination of the nucleic acids because post-amplification manipulation is avoided [3,19].

In this real-time PCR study, HCMV and EBV DNA were detected in 16.7% (3/18) and 5.6% (1/18) of the acute apical abscess samples, respectively. Using reverse-transcription PCR, Sabeti and Slots [15], found that all symptomatic periapical lesions contained either HCMV or EBV, and they identified dual infection by the two viruses in 69.6% of symptomatic lesions. Using single and nested PCR, Ferreira et al. [2], did not find HCMV or EBV in their abscess specimens. In another study, Ferreira et al. [18] found EBV in 6% of purulent exudates from acute apical abscesses, whereas they did not identify HCMV in abscess samples using single and nested PCR. The discrepancies in the findings among our study and the three studies mentioned above might arise for several reasons, including differences in the primers used, methods used for DNA extraction, and host and environmental factors such as genetic background, ethnicity, and socioeconomic status [8,20–23]. We used same sampling techniques applied by Chen et al. [1]. This may be the reason of our results consistency with the findings of Chen et al. [1], who found HCMV and EBV in 29 and 6.5% of abscess specimens, respectively.

In this study, when pairs of the target species were evaluated, the pair *P. gingivalis*/*T. denticola* was detected in two cases (11%), *P. gingivalis*/*T. forsythia* in one case (5.6%), and *T. denticola*/*T. forsythia* in two cases (11%). Ferreira et al. [18], reported that several bacterial pairs showed moderately positive associations: *Porphyromonas endodontalis*/*Filifactor alocis*; *F. alocis*/*Pyramidobacter piscolens*; *Dialister pneumosintes*/*P. piscolens*; *Olsenella uli*/*P. piscolens*; and *P. endodontalis*/*O. uli*. The differences might occur for several reasons, such as differences in the primers used and methods of DNA extraction, differences in the endodontic microbiota, and several host

and environmental factors, such as genetic background, ethnicity, socioeconomic status, psychologic stress, smoking, and the nature of the species colonizing other individuals in the same country [11,24,25].

It has been suggested that the viral-bacterial coinfection observed in abscesses has two basic interpretations: viruses impair local host defences, favouring bacterial overgrowth, or the occurrence of viruses is just an epiphenomenon of the bacterial infection that caused inflammation, with a consequent influx of virus-infected inflammatory cells to the area [18]. In this study, viral-bacterial associations involving the target bacteria and viruses were observed in two samples (11%). The pairs HCMV/*T. denticola* and HCMV/*T. forsythia* were each detected in 1 of 18 (5.6%) abscess specimens. Using single or nested PCR, Ferreira et al. [18], found that bacterial and viral DNA occurred concomitantly in two-thirds of the samples from endodontic abscesses. They found that human herpesvirus-8 and human papillomavirus were the most prevalent viruses showing associations with the target bacterial species. The lower prevalence of viral-bacterial associations in our study may have resulted from the clinical status of the study subjects, viral diagnostic methods used, or geographic differences in herpes viral occurrence [3,8,11,19,24].

## Conclusion

Our PCR-based findings revealed that HCMV was the most frequent herpesvirus and *T. denticola* was the most frequent bacterium among the target organisms in acute apical abscesses. Bacterial coinfections were found in four samples (22%). The pairs HCMV/*T. denticola* and HCMV/*T. forsythia* were detected in one case each. Additional studies using *in vitro* systems or animal models are required to elucidate the role of herpesviruses in the pathogenesis of periapical pathosis.

## Clinical Significance

The present bacterial and viral findings may have future therapeutic relevance for periapical abscesses and other periapical pathosis.

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