Detection of *Treponema Denticola* in Symptomatic Apical Periodontitis and in Symptomatic Apical Abscesses by Real-Time PCR

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ABSTRACT

Objectives: The aim of this study was to investigate the presence of *Treponema denticola* in symptomatic apical periodontitis and in symptomatic apical abscesses by real-time polymerase chain reaction (PCR) method.

Methods: Microbial samples were collected from 60 single-rooted teeth having carious lesions and necrotic pulps. For each tooth, clinical data including patient symptoms were recorded. Teeth were categorized by diagnosis as having symptomatic apical periodontitis or symptomatic apical abscess. Aseptic microbial samples were collected using paper points from 30 infected root canals and from aspirates of 30 abscesses. DNA was extracted from the samples by using a QIAamp® DNA mini-kit and analyzed with real-time PCR.

Results: *T. denticola* was detected in 24 of 30 cases diagnosed as symptomatic apical abscesses (80%), and 19 of 30 cases diagnosed as symptomatic apical periodontitis (63.3%). In general *T. denticola* was found in 43 of 60 cases (71.6%).

Conclusions: Our findings suggest that *T. denticola* can participate in the pathogenesis of symptomatic apical abscesses. (Eur J Dent 2009;3:107-113)

Key words: *Treponema denticola*; Symptomatic apical abscess; Necrotic tooth; Root canal; Real-time PCR.

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INTRODUCTION

The association of spirochetes with oral diseases has been known for many years. Amongst the oral spirochetes, nine species have been cultured and named. Four species have been cultured widely and reliably by many laboratories: Treponema denticola, *T. pectinovorum, T. socranskii, T. vincentii.*[1] Cultivation of oral spirochetes is difficult because of the unique and complex nutritional requirements and strict anaerobic conditions needed for growth. Despite recent developments of improved culture

conditions and artificial media, many spirochetes have still resisted efforts for long-term in vitro cultivation.[2]

T.denticola is a gram-negative, anaerobic, helically coiled, highly motile bacterium. This bacterium is by far the best characterized of the currently cultivable oral spirochetes species. Nevertheless, it is usually difficult to grow.[3,4]

More than a hundred years ago, Miller[5] reported the occurrence of spirochetes in endodontic infections suggested that these bacteria were involved in the pathogenesis of pulpitis and apical periodontitis. Spirochetes have then been found in necrotic root canals using microbiological methods,[6,7] dark field microscopy[8,19] and transmission electron microscopy.[10] Together, these publications suggest that spirochetes were only occasionally found and when they occurred, made up a small proportion of the flora.[11]

The advent of molecular genetic technology for microbial detection and identification has sparked a better understanding of the roles that difficult-to-grow or not-yet-cultivated bacteria, including spirochetes, play in diverse polymicrobial infections, including periradicular diseases.[12,13] The application of molecular diagnostic methods for identification of spirochetes has demonstrated that occurrence of these spiral bacteria in infection of endodontic origin has been overlooked by technical hurdles of cultivation techniques.[14] About 90% of the samples from different types of endodontic infections have been demonstrated to harbor at least one species of spirochetes.[1,15,16]

The molecular methods most often used for microbial identification are the polymerase chain reaction (PCR) method and its variations.[17] The PCR is a simple method for amplification of specific segments of DNA or cDNA reverse transcribed from RNA.[18] Numerous variations in the standard PCR procedure have been developed. Single PCR, nested PCR, reverse transcriptase PCR, multiplex PCR, and real-time PCR are examples of approaches commonly used to identify target species.[17] Most PCR assays are qualitative or can be semi-quantitative. One exception is the real-time PCR, which is characterized by the continuous measurement of products throughout the reaction.[13] Real-time PCR assays allow the quantification of individual target species and total bacteria in clinical samples.[17]

Periradicular lesions are classified into five main groups: acute apical periodontitis, chronic apical periodontitis, condensing osteitis, acute apical abscess, and chronic apical abscess. Teeth associated with significant symptoms such as pain or swelling are referred to as symptomatic (acute), whereas those with mild or no symptoms are identified as asymptomatic (chronic).[19]

Symptomatic apical periodontitis is localized inflammation of the periodontal ligament in the apical region.[20] A tooth with a symptomatic apical periodontitis will give a very painful response to biting pressure or percussion. This tooth may or may not respond to pulp vitality tests, and the radiograph of this tooth will generally exhibit widened periodontal ligament space but no periradicular radiolucency.[21] Symptomatic apical abscess is an inflammatory process in the periradicular tissues of teeth, accompanied by exudates formation within the lesion.[20] A tooth with a symptomatic apical abscess will be very painful to biting pressure, percussion, and palpation. This tooth will not respond to any pulp vitality tests and will exhibit varying degrees of mobility, and the radiograph can exhibit anything from a widened periodontal ligament space to periradicular radiolucency. Swelling will be present in the mucobuccal fold and facial tissues adjacent to the tooth.[21]

Due to difficulties in isolating and identifying of oral treponemes, the present study was undertaken to determine the presence of *T.denticola* in microbiological samples taken from symptomatic endodontic cases using a sensitive molecular method, 'real-time PCR'.

MATERIALS AND METHODS

Patients and sampling

Specimens were selected from adult patients that had been referred for endodontic treatment to the Department of Endodontics, Dental School of Ataturk University (Erzurum, Turkey). Medical histories revealed that all patients were in good general health, and had no important systemic diseases such as diabetes. Patients that had received antibiotic therapy during the last 3 months were excluded from the study. The ages of the patients ranged from 18 to 45 years. Approval to undertake the study was given by the Ethical Committee in Research of the Dental School of

Ataturk University and informed consent was obtained from all patients.

The following clinical features of each tooth were recorded: the type of restoration, pain on occlusion, tenderness to percussion or palpation, swelling, and the presence of periapical radiolucency. Cases with a periodontal pocket probing depth greater than 4 mm and teeth in which proper rubber dam isolation could not be achieved were excluded from the study.

Sixty single-rooted teeth, all of them having carious lesions and necrotic pulps were included in this study. Of these, thirty teeth showed both spontaneous symptoms and tenderness to percussion and thickening of the periodontal ligament space on radiographic examination were diagnosed as symptomatic apical periodontitis.[21] The other thirty teeth showed tenderness to percussion and palpation, swelling, mobility and radiographic evidence of periradicular bone loss were diagnosed as symptomatic apical abscesses.[21]

Samples from cases of symptomatic apical periodontitis were obtained from the root canals using strict asepsis as described previously.[3,22] Briefly, teeth were cleansed with pumice, isolated with rubber dam, and the surrounding field 3% hydrogen peroxide and then decontaminated with 3% hydrogen peroxide and then decontaminated with a 2.5% sodium hypochlorite solution. Access preparations were made using sterile burs without water spray. The operative field, including the pulp chamber, was then swabbed with 2.5% sodium hypochlorite. This solution was inactivated with sterile 5% sodium thiosulfate. Samples were initially collected by means of a #15 K-type file (Maillefer, Ballaigues, Switzerland) with the handle cut off. The file was introduced to a level approximately 1mm short of the tooth apex, based on diagnostic radiographs, and a discrete filing motion was applied. Afterwards, two sequential paper points were placed to the same level and used to soak up the fluid in the canal. Each paper point was retained in position 1 min the cut file and two paper points were then transferred to cryotubes containing 1mL of 5% dimethyl sulfoxide (DMSO) in trypticase soy broth (TSB). Samples were then immediately frozen at –20ºC.

Samples from cases of symptomatic apical abscesses were obtained by aspiration of purulent exudates from the swollen mucosa over each abscess. After disinfection of the overlying mucosa with 2% chlorhexidine, a sterile disposable syringe was used to aspirate the pus, which was immediately injected into cryotubes containing 1 ml of 5% dimethyl sulfoxide (DMSO) in trypticase soy broth (TSB).[16] Samples were then immediately frozen at –20ºC.

DNA extraction

The frozen samples in 2 mL collection tube with TSB-DMSO were left at room temperature for 20 min. After the temperature of the samples had been adjusted to room temperature, they were vigorously vortexed, centrifuged at 8,000 x g for 5 min, then the supernatants were removed and the pellets were used[23] for DNA extraction by using a QIAamp® DNA mini-kit (QIAGEN®, GMBH, Germany). The protocol recommended by the kit manufacturer for DNA extraction from the tissue samples was followed precisely.

Oligonucleotide primers

The set of the species-specific primers and the TaqMan probe of real-time PCR amplification for T. denticola were designed as described previously by Yoshida et al.[24] Briefly, a fluorescent probe was used to monitor PCR product formation continuously. The oligonucleotide probe was labeled at the 5'-end with a reporter dye (FAM: 6-carboxyfluorescein) and at the 3'-end with quencher dye (TAMRA: 6-carboxytetramethylrhodamin). The primer-probe set comprised a forward primer (5'-AgAgcAAgcTcTcccTTAccgT-3') and a reverse primer (5'-TAAgggcggcTTgAAATAATgA-3') and a probe (5'-FAM-cAgcgTTcgttCTgAgccAggATcA-TAMRA-3'). The ubiquitous primers that match almost all bacterial 16S rRNA genes at the same position but not 18S rRNA gene from eukaryotic cells were used as a positive control for the DNA extraction and probable PCR inhibitors. A pair of ubiquitous primers was 5'—3' gAT TAg ATA ccc Tgg TAg Tcc Ac and ccc ggg AAc gTA TTc Acc g (base positions were 796-818 and 1381-1399, respectively). The amplicon size was 604 bp.[4]

PCR amplification

The amplification and detection of DNA with species-specific primers by real-time PCR were performed with the ICycler IQ Multicolor

Real-Time RCR Detection System (BIO-RAD® Laboratories, Inc. Hercules, CA USA). For each real-time PCR, 20 µL of a mixture that contained 1X QuantiTect Probe PCR buffer compositing of Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7, dNTPs (contains ultra pure quality dATP, dGTP, dCTP and dTTP/dUTP) (QIAGEN® GMBH, GERMANY) 0.7 µM for each forward and reverse primers and 0.24 µM for the dual labeled probe, 5 µL of target DNA from samples were used. Totally, 25 µL 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, Inc. Hercules, CA USA). The DNA amplification condition was used as following; 1 cycle of 50ºC for 2 min, 1 cycle of 95ºC for 15 min, followed by 50 cycles of 95ºC for 30 s and 58ºC for 90 s, and infinite hold for 11ºC.

RESULTS

All amplifications were obtained using ubiquitous primers, suggesting that there was not any PCR inhibitor in the DNA samples used (Figure 1).

PCR amplifications with species-specific primers gave the similar results when they were repeated. 68ºC of melting temperature for the PCR product obtaining with species specific primers was used to establish positive results. Also 58ºC of melting temperature was proved by amplification of DNA from *T. denticola* used as positive control DNA.

In general, real-time PCR method enabled the detection of *T. denticola* in 43 of 60 symptomatic endodontic cases (71.6%). *T. denticola* was detected in 24 of 30 cases diagnosed as symptomatic apical abscesses (80%), and 19 of 30 cases diagnosed as symptomatic apical periodontitis (63.3%). Data regarding prevalence values are presented in Figure 2.

DISCUSSION

The development of effective strategies for root canal therapy is dependent upon understanding the composition of the pathogenic flora of the root canal system. Identification of the root canal isolates from previous studies has traditionally been performed using standard microbiological and biochemical techniques.[25] Data on microbial morphology provides few clues for the identification of most microorganisms, and physiological traits are often ambiguous.[26,27] In addition, several microorganisms are difficult or even impossible to grow under laboratory conditions.[26] These factors are especially true in the case of spirochetes.[1,12]

Recent studies using sensitive molecular diagnostic methods have allowed detection of microorganisms that are difficult or even impossible to culture in infections elsewhere in the human body, including within the root canal system.[28] PCR techniques have been increasingly used in investigations of the periodontal and root canal flora and are able to detect the presence of genomic DNA of bacteria present in the root canal space with a high degree of sensitivity and specificity.[29,30] The real-time PCR method used in this study was a powerful technique combining sample amplification and analysis in a single reaction tube.[31] The advantages of real-time PCR are the rapidity of the assay, the ability to quantify and identify PCR products directly without the use of agarose gels, and the fact that contamination of the nucleic acids is limited because of avoidance of post-amplification manipulation.[32]

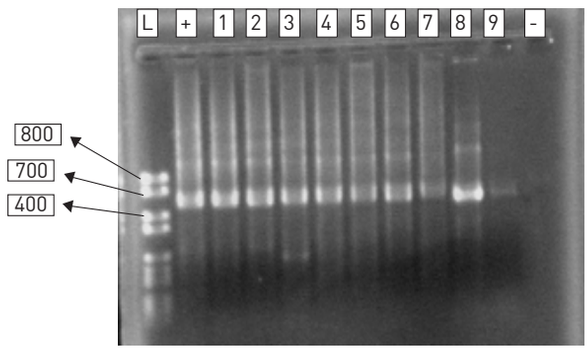

Figure 1. PCR amplicons of nine samples obtained by ubiquitous primers in 1% agarose gell.

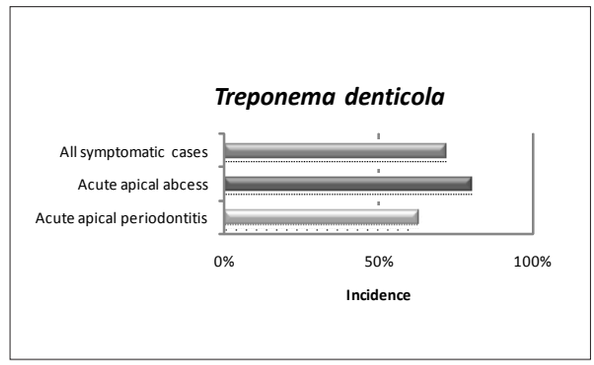

Figure 2. Incidence of *T. denticola* in symptomatic endodontic cases.

The polymicrobial nature of the endodontic microbiota suggests that bacteria are interacting with one another and such interaction can play an important role for both survival and virulence.[33] In a mixed bacterial community, it is likely that *T. denticola* has its virulence enhanced or it can enhance the virulence of other species in the consortium.[34] Oral treponemes can cause abscesses when inoculated in experimental animals.[35] These microorganisms are reported to possess an array of putative virulence traits that may be involved in the pathogenesis of endodontic abscesses by wreaking havoc on host tissues and/or by allowing the microorganism to evade host defence mechanisms.[16] Its virulence factors include the major surface protein, the chymotrypsin-like protease complex, other extracellular or membrane-associated proteolytic and hydrolytic enzymes, lipoligosaccharide, lipoproteins, phospholipases, and metabolic end products (acetate, lactate, ammonia, and hydrogen sulphisde).[35]

In order to identify microorganisms and study their characteristics and pathogenic potential, it is essential that accurate methods be used for sampling.[11] Contamination of the samples is a significant problem to be considered in clinical studies.[4] In our study, we used sampling methods described previously[3,16,22] to reduce the risk of contamination in cases with symptomatic apical periodontitis and symptomatic apical abscesses.

In the present study, *T. denticola* was detected in 80% of cases diagnosed as symptomatic apical abscesses, and 63.3% of cases diagnosed as symptomatic apical periodontitis. The differences between prevalence values of *T. denticola* may probably have been because of different environmental conditions (e.g. oxygen tension, nutrient availability, bacterial interactions).[11] Siqueira et al[4] found T. denticola in 52% of symptomatic cases by means of single PCR assay. Siqueira et al,[36] in another study, found this bacterium in 4% of samples from acute periradicular abscesses by means of checkerboard DNA-DNA hybridization. Jung et al[37] have not found this bacterium in periradicular lesions using PCR amplification with bacterial universal primers and subsequent dot-blot hybridization. The disagreements in findings between our study and the three studies above mentioned can be explained by the different detection limits of the methods used. The real-time PCR used in the present study is more sensitive both single PCR assay and DNA-DNA hybridization methods.

Our finding disagrees with the findings reported by Rôças et al[1] and Baumgartner et al.[2] Rôças et al[1] reported the presence of *T. denticola* in 80% of samples from acute apical periodontitis using nested PCR. Baumgartner et al[2] reported the finding of *T. denticola* in 34.5% of samples from abscesses/cellulitis using nested PCR. Such discrepant findings may be caused by different molecular techniques employed or geological difference.[38] Some researchers,[16,39,40] using molecular methods, found *T. denticola* in 79, 78, and 75% of cases diagnosed as symptomatic periradicular abscesses, respectively, supporting our results.

CONCLUSIONS

Our findings suggest that *T. denticola* can participate in the pathogenesis of symptomatic apical abscesses. Further investigations using animal model are required to further evaluate the mechanism of *T. denticola* pathogenicity. PCR based molecular techniques, even though expensive are very convenient for detection of *T. denticola* in dental and dental related samples.

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