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Detection of selected anaerobic pathogens in primary and secondary endodontic infections in a Turkish population

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The aim of this investigation was to examine the presence of 8 bacterial anaerobic species in endodontic samples from patients with primary and secondary infection. The association of clinical signs and symptoms with constituent species were also evaluated. Microbial samples were obtained from 72 teeth with primary endodontic infection and 35 teeth with secondary endodontic infection. DNA was extracted from samples and analyzed with a polymerase chain reaction (PCR)-based identification assay. Medical and dental histories were obtained from each patient. The prevalence of the targeted bacterial species was recorded for each case and descriptive statistical analyses were performed using the Pearson Chi-squared test. Nucleid acid amplification method (NAAM) analysis showed that all specimens were positive at least for 1 or more samples in primary and secondary infection teeth. The most frequently detected bacteria in all speciemens were *Porphyromonas gingivalis*, followed by *Porphyromonas micros, Porphyromonas endodontalis, Fusobacterium nucleatum, Porphyromonas intermedia* and *Tannerella forsythia*, respectively. The percentages of all selected bacteria found in primary infection group were higher than secondary infection group except for *Porphyromonas intermedia*. However, statistically significant difference was found only for *T. forsythia* and *F. nucleatum* which were higher percentage in primary infection than in secondary infection group. There was a significant association between tenderness to percussion and *P. gingivalis* ($p < 0.05$), pain with *Porphyromonas melaninogenica* ($p < 0.05$) and swelling with both *P. gingivalis* ($p < 0.05$) and *P. melaninogenica* ($p < 0.05$). Findings indicated that the prevalence of some species found in the primary infection group were higher than in the secondary infection group. In this study there was a significant association between tenderness to percussion and *P. gingivalis*, pain with *P. melaninogenica* and swelling with both *P. gingivalis* and *P. melaninogenica*.

Key words: Primary endodontic infection, secondary endodontic infection, anaerobic bacteria, nucleid acid amplification method (NAAM).

INTRODUCTION

Apical periodontitis is caused by bacteria of infected root canals (Kakehashi et al., 1965). Necrotic root canals are typically polymicrobial, with nearly equal proportions of Gram-positive and Gram-negative bacteria, and are dominated by anaerobic bacteria (Siqueira, 2002). In contrast, the microbial flora in secondary endodontic infec-

tions have been described as monoinfections or infections including a few Gram-positive bacterial species, with approximately equal proportions of facultative and obligate anaerobes (Sundqvist et al., 1998; Pirani et al., 2008).

Infections of the root canal system with facultative and obligate anaerobic bacteria have been associated with different clinical signs and symptoms (Jung et al., 2000; Gomes et al., 2004; Siqueira et al., 2004; Cavrini et al., 2008). Significant associations were found between individual clinical features and the following pairs of species: *Peptostreptococcus* spp*., Prevotella melaninogenica, P. micra* are associated with pain, *P. micra and Prevotella* spp*.* is associated with swelling and *Prevotella* spp*., Eubacterium* spp*. and Peptostreptococcus* spp*.* are associated with wet canals (Gomes et al., 1996). It is well known that most periodontal pathogens like *P. gingivalis* and *P. endodontalis* are also endodontic pathogens which are the key organisms in adult periodontitis and frequently found in root canal infections. (van Winkelhoff et al., 1985). *T. forsythia* is strongly associated with chronic periodontitis, an inflammatory disease of the tooth-supporting tissues, leading to tooth loss (Settem et al., 2012). *F. nucleatum* appears to be associated with the development of the most severe forms of inter appointment endodontic flare-ups (Chávez de Paz Villanueva, 2002). *P. melaninogenica* and *P. micra* is associated with pain and swelling (Drucker, 2000).

Recent findings revealed differences in the prevalence of several species between distant geographical locations (Baumgartner et al., 2004; Siqueira et al., 2005). Studies investigating the polymicrobial etiology of apical periodontitis indicated that the bacterial community profiles significantly vary between patients of different locations (Baumgartner et al., 2004; Siqueira et al., 2005). Until now, no previous investigation has been reported the presence of obligate anaerobic bacteria in endodontic samples from the root canal microbiota of patients from Cukurova region of Turkey.

The purpose of this investigation was to examine the presence of 8 bacterial anaerobic species (*Fusobacterium nucleatum, Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella intermedia, Prevotella melaninogenica, Prevotella nigrescens, Tannerella forsythia, Parvimonas micra*),which are associated with endodontic infections, from patients with primary infection and secondary infection by employing Nucleic acid amplification methods (NAAM) and the association of clinical signs and symptoms with the constituent species.

MATERIALS AND METHODS

One-hundred and seven patients, who were referred to Cukurova University Dental School for endodontic treatment or retreatment were included in the study. Seventy-two teeth presented necrotic pulp and 35 teeth had previously been root-filled and showed clinic and radiographic evidence of apical periodontitis. All patient related procedures used in this study were approved by the Ethical Committee of the University of Cukurova and informed consent was obtained from each patient.

Clinical signs and symptoms

Medical and dental histories were obtained from each patient. Age, gender, tooth type, pulp status, pain, history of previous pain, tenderness to percussion, pain on palpation, mobility, presence of a sinus, presence of swelling, history of previous and present antibiotic therapy were recorded. Periodontal probing depths of selected teeth were also recorded and periodontal pockets more than 4 mm deep were excluded from the study because of possible endodontic-periodontal infection. For all teeth the presence of periapical radiolucency was assessed using the periapical index (PAI), determined with a paralleling X-ray technique (Orstavik et al., 1986). Patients who had not been treated with antibiotics in the preceding 3 months and who had no systemic diseases were included in the study. For necrotic teeth, an electric pulp test was conducted.

Sampling procedures

For sampling, each tooth was cleaned with pumice and isolated with a rubber-dam. Gingival barier 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 (NaOCl) solution. After disinfection, the coronal restorations were removed. Endodontic access was completed with a sterile high speed carbide bur. After completion of the endodontic access, 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. A microbiologic sample was taken from the root canal after discrete filing motion with sterile #15 K-file and three sterile paper points. If the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Afterwards paper points were placed into the canal, with each left for 1 min for absorbing all the fluids present within them. These paper points were then transferred to cryo-tubes containing TE buffer and immediately frozen at -80ºC.

In cases with secondary infections, pre-existing root canal fillings were removed using a Gates-Glidden drill and the apical material was retrieved using K-type files without the use of chemical solvents. Sterile saline solution was introduced into the canal to remove any remaining materials and to release the debris. The same procedure was used for the root canal sampling.

Polymerase chain reaction (PCR) assays

DNA extraction of samples was performed using the Invitrogen PureLink Genomic DNA Mini Kit (Lot No: 449092, Carlsbad, CA 92008) according to the manufacturers recommendations. Reference DNA from the selected microorganisms: *Fusobacterium nucleatum-ATCC 25586, P. gingivalis-ATCC 33277, P. endodontalis-ATCC 35406, P. intermedia-ATCC 15032, P.melaninogenica-ATCC 25845, P.nigrescens-ATCC 33563, T. forsythia-ATCC 43037 P. micra-ATCC 33270* was extracted. A negative control (without DNA) and a positive control (DNA of

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Table1. PCR primers, expected amplicon sizes and thermocycling conditions for endodontic pathogens.

Target microorganism	Primer sequences (from 5'to 3')	Amplicon size (bp)	Thermocycling conditions	Cycle
Fusobacterium nucleatum	AGA GTT TGA TCC TGG CTC AG GTC ATC GTG CAC ACA GAA TTG CTG	360bp	94°C 1 min, 60°C 1 min, 72°C 2.5 min	30
Porphyromonas gingivalis	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404bp	94°C 30 s, 60°C 1 min, 72ºC 2 min	36
Porphyromonas endodontalis	GCT GCA GCT CAA CTG TAG TC CCG CTT CAT GTC ACC ATG TC	672bp	94°C 30 s, 60°C 1 min, 72 ºC 2 min	36
Prevotella intermedia	TTT GTT GGG GAG TAA AGC GGG TCA ACA TCT CTG TAT CCT GCG T	575bp	95°C 30 s, 55°C 1 min, 72ºC 2 min	36
Prevotella melaninogenica	CGT CAT GAA GGA GAT TGG ATA GAA CCG TCA ACG CTC	122bp	95°C 15 s, 54°C 30 s, 72ºC 1 min	25
Prevotella nigrescens	ATG AAA CAA AGG TTT TCC GGT AAG CCC ACG TCT CTG TGG GCT GCG A	804bp	94°C 30 s, 55°C 1 min, 72ºC 1 min	36
Tannerella forsythia	GCG TAT GTA ACC TGC CCG CA TGC TTC AGT GTC AGT TAT ACC T	641bp	94°C 30 s,55°C 30 s, 72ºC 1 min	35
Parvimonas micra	AGA GTT TGA TCC TGG CTG AG ATA TCA TGC GAT TCT GTG GTC TC	207bp	94°C 1 min, 60°C 1 min, 72°C 2.5 min	30
Universal 16S rDNA	GAT TAG ATA CCC TGG TAG TCC AC CCC GGG AAC GTA TTC ACC G	602bp	95°C 30 s, 60°C 1 min, 72°C 1 min	36

Fusobacterium nucleatum, P. gingivalis, P. endodontalis, P. intermedia, P. melaninogenica, P. nigrescens, T. forsythia, P. micra) were used. The DNA concentrations of reference strains and clinical endodontic samples were assessed by spectrophotometer at the 260 nm absorbance level.

Table 1 lists the PCR primers, predicted amplicon lengths and thermocycling conditions for the bacterial species tested. Initially, a universal eubacterial primer pair was used to detect DNAs from all bacterial species present in the sample (Dahlen et al., 2000; Ashimoto et al., 1996). The primers were purchased from Genoks Technology-Ankara. PCR reactions were performed in a total volume of 50µl containing 1.25 U Taq DNA polymerase, 2 µl- 5 µM $MgCl_2$, 5 µl-10X Tris-HCl (Vivantis, PL 1202), 0.2mM of each deoxynucleoside triphosphates, and a specific primer pair. The concentration of each primer was 0.5 µM for all target bacteria. DNA amplification was performed in a thermal cycler (BIO-RAD, MJ Mini Personal Thermal Cycler). PCR products were stored at -80^{o}C.

The amplification products were analyzed by 1.8% agarose gel electrophoresis containing 0.5% ethidium bromide in TBE buffer (Tris-borate EDTA) at 100 V for 1 h and visualized under ultraviolet light. The identity of each band was determined in comparison using a 100-bp DNA ladder.

Statistical analyses

Data collected for each case were recorded on an electronic spreadsheet and statistically analyzed by using SPSS 12.0 (SPSS Inc., Chicago, IL). The prevalence of the target bacterial species was recorded as the percentage of the cases examined. Descriptive statistical analyses were performed using the Pearson Chi-squared test.

RESULTS

None of the specimens revealed negative bacterial DNA that was amplified by using universal eubacterial primers. The incidence of detection of the selected bacterial species for all samples is summarized in Figure 1.

The results of NAAM analysis showed that all specimens were positive at least for 1 or more samples in the primary and secondary teeth infection. The most frequently detected bacteria in all specimens was *P. gingivalis*, followed successively by *P. micra, P. endodontalis, F. nucleatum, P. intermedia* and *T. forsythia* (Table 2). The percentage of all selected bacteria found in the primary infection group was higher than the secondary infection group except for *P. intermedia*. However, statistically significant difference was found only for *T. forsythia* and *F. nucleatum* ($p<0,05$), which were both higher in the primary infection compared to the secondary infection group.

In the primary infection group 46 out of 72 (63.9%) samples consisted three or more species per canal whereas this ratio was lower in the secondary infection

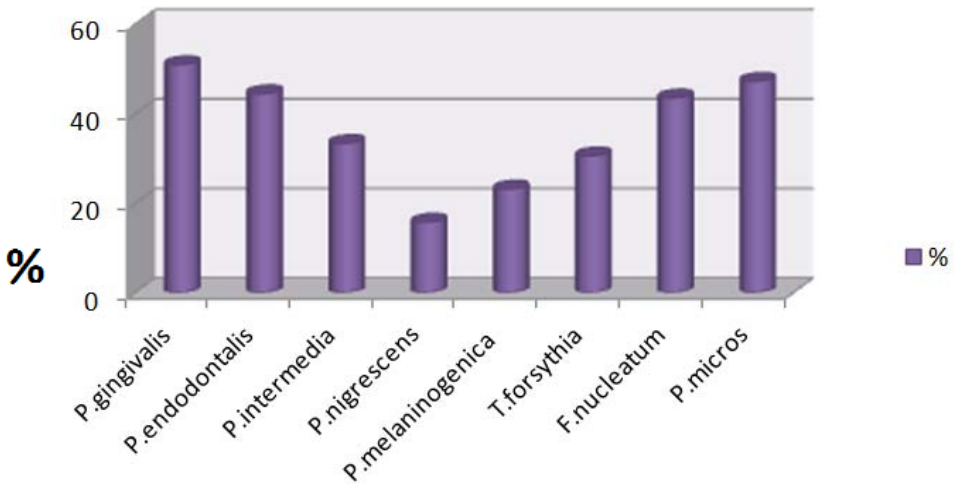

Figure 1. Incidence of bacteria in all specimens.

Table 2. The incidence of bacteria detected in the primary and secondary endodontic infections.

Target bacteria	Primary endodontic infection		Secondary endodontic infection infection Failed endodontic treatment	
		%		%
Porphyromonas gingivalis	39/72	54.2	16/35	45.7
Porphyromonas endodontalis	35/72	48.6	13/35	37.1
Prevotella intermedia	22/72	30.6	14/35	40
Prevotella nigrescens	12/72	16.7	5/35	14.3
Prevotella melaninogenica	18/72	25	6/35	17.3
Tannerella forsythia	28/72	38.9*	5/35	14.3
Fusobacterium nucleatum	38/72	52.8†	9/35	25.7
Parvimonas micra	35/72	48.6	15/35	42.9

* *p*: 0.008 † *p*: 0.010.

Table 3. The incidence of bacterial combinations in specimens of the primary and secondary endodontic infections.

Bacterial combination	N	%
P.intermedia + *P.micra*	30/107	28
F.nucleatum + *P.micra*	28/107	26.2
P.endodontalis + *P.gingivalis*	26/107	24.3
P.endodontalis + *P.micra*	24/107	22.4
T.forsythia + *P.gingivalis*	23/107	21.5
P.micra + *P.gingivalis*	23/107	21.5
F.nucleatum + *P.gingivalis*	19/107	17.8
T.forsythia + *P.micra*	19/107	17.8
F.nucleatum + *P.intermedia*	16/107	15

group (34.3%) and this difference was statistically significant (p < 0.05). As for the specimens containing only one targeted bacteria, the secondary infection group had a higher incidence (31.4%) than the primary infection group (18.1%). However, there was no statistically significant difference between two groups (p>0.05).

In the present study, the combination of bacterial species were also investigated and *P.micra* was found together with the species *P. intermedia, F. nucleatum, P. endodontalis, P. gingivalis and T.forsythia* in all specimens with the incidences of 28, 26,2, 22,4, 21,5 and 17,8%, respectively. Table 3 shows the most prevalent bacterial combinations in all specimens tested.

Eighty-six of 107 (80%) samples had symptomatic root canal infections and the association was found between spontaneous pain and primary infection group (62/72). This ratio demonstrated a statistically significant (p < 0.05) relationship between clinical signs and symptoms and the primary infections. The 60.5% of symptomatic cases harbored 3 or more of the tested endodontic pathogens, which could point out the relationship between bacterial complexity and clinical characterization of the infection (p < 0.05). Table 4 shows the prevalence of micro-

Table 4. The prevalence of microorganisms associated with the clinical signs and symptoms of infected root canals.

Target microorganism	N		Pain	Previous pain	Tenderness to percussion	Swelling	Periapical radiolucency	Fistula
P. gingivalis	55	N	26	10	37§	32†	17	41
		%	47.3	18.2	67.3	58.2	30.9	74.5
P. endodontalis	48	N	22	10	24	18	13	28
		%	45.8	20.8	50	37.5	27.1	59.6
P. intermedia	36	N	19	5	16	14	11	28
		%	52.7	13.9	44.4	38.8	30.6	77.7
P. nigrescens	17	N	8	2	10	8	2	11
		%	47.05	11.8	58.8	47.1	11.8	64.70
P.melaninogenia	24	N	19*	3	14	14‡	11	19
		%	79.2	12.5	58.3	58.3	45.8	79.1
T. forsythia	33	N	11	7	18	14	14	22
		%	33.3	21.2	54.5	42.4	42.4	66.6
F. nucleatum	47	N	20	9	26	21	15	34
		%	42.6	19.1	55.3	44.7	31.9	72.3
P. micra	51	N	20	5	21	21	14	37
		%	40	10	42	42	28	74

* p: 0.000, † p: 0.018, ‡ p: 0.022, § p: 0.001.

organisms associated with clinical signs and symptoms in 107 infected root canal samples. There was a significant association between tenderness to percussion and *P. gingivalis* ($p < 0.05$), pain with *P. melaninogenica* ($p < 0.05$) and swelling with both *P. gingivalis* ($p < 0.05$) and *P. melaninogenica* ($p < 0.05$).

DISCUSSION

The purpose of this study was to evaluate the presence of selected bacterial pathogens by using both universal and specific PCR primers in the primary and secondary root canal samples and to associate these species with clinical signs and symptoms. In this study *P. gingivalis* was the most frequently detected bacteria in all specimens both from patients with primary and secondary root canal infections. The black-pigmented species studied were detected at a higher frequency in teeth with necrotic pulp than in teeth with failing endodontic treatment. In accordance with this study, Gomes et al. (2005) reported that *P. gingivalis, P. endodontalis, P. intermedia* and *P. nigrescens* were detected more frequently in untreated teeth with necrotic pulp than in teeth with failing endodontic treatment. Blome et al. (2008) reported *P. endodontalis* as the prevalent microorganisms in primary and secondary endodontic infections but in contrast with our finding they found *P. gingivalis* in low ratios. *P. endodontalis* has been almost particularly associated with endodontic infections, and its pathogenicity depends on the presence of the other species in a consortium (van Winkelhoff et al., 1992).

F. nucleatum was found as the second prevalent bacteria in primary endodontic group and also was found in low ratios in secondary infection group. *F. nucleatum* was previously shown to increase pathogenicities of other organisms in mixed culture, especially those of *P. gingivalis* and *P. intermedia* (Baumgartner and Falker, 1991, Siqueira et al., 2000). *F. nucleatum* and *P. gingivalis* have also been described as common endodontic bacterial pathogens in other study. (Podbielski et al., 2003). In accordance with our study, Vianna et al. detected *F. nucleatum, T. forsythia, P. gingivalis* frequently in necrotic root canal by the DNA chip (Vianna et al., 2005).

Fouad et al. (2002) demonstrated that *P. nigrescens* is more prevalent in endodontic infections than *P. intermedia*. In contrast, in our study *P. intermedia* ratio is higher than *P. nigrescens*. Tomazinho and Avila-Campos (2007) found that *P. gingivalis* and *P. nigrescens* were the most prevalent, followed by *P. intermedia* and *P. endodontalis* in 60 PCR samples taken from chronic endodontic infections. *P. endodontalis* and *P. gingivalis* have been consistently encountered in endodontic infections, and attributed a role for both in the etiology of acute abscess

(van Winkelhoff et al., 1985; Sundqvist et al., 1989).

T. forsythia had never been detected in root canals by culture but is confirmed that this organism is a common member of the microbiota associated with different types of primary infections including abscess by molecular biology approaches (Fouad et al., 2002; Siqueira and Roças, 2003). In this study, it is of interest that a gram negative rod, *T. Forsythia*, was detected in 33 of 107 root canal samples and that in 30 of these 33 samples, this bacterium was always associated with one or more members of the black pigmented gram negative rods. In accordance with this study, *T. forsythia* was found in root canals from 40 to 59.1% and this species is suggested to play a major role in the pathogenicity of primary endodontic infections (Siqueria and Roças, 2003; Blome et al., 2008).

Bacterial combinations in root canals may be more pathogenic than individual strains (Fabricus et al., 1982). Therefore, it is important to determine the association of bacterial combinations with clinical signs and symptoms or treatment outcome, as well as the association of certain microorganisms with each other. In accordance with some previous studies reporting the positive ecological relationship between *P. gingivalis* and *T. forsythia*, these two bacteria were found together in 23 of 107 samples in our study (Jung et al. 2000, Roças et al., 2001). Moreover, in 30 of 107 endodontic samples *P. intermedia and P. micra* combination was detected. In all tested specimens, *P. micra* had higher combination ratios compared to the other anaerobic species. *P. micra* is also detected in 28 of 107 samples with *F. nucleatum*, that is concordant with the results reported by Blome et al. (2008) but further research is needed to confirm these bacterial relations.

Our results demonstrated that *P. gingivalis* was associated with symptoms of tenderness to percussion and swelling. Jacinto et al. (2003) found a relation between pain on palpation and *P. gingivalis* and *Peptostreptococcus* spp. And similar to our study Siqueira et al. (2000) found six bacterial species which were detected in teeth tender to percussion, of which *P. gingivalis* and *P. micra* were the predominant species.

The disparity in the composition of root canal microbiota can be related to sampling method, different methodological techniques, and other factors including geographical effects (Dumani et al., 2012). Baumgartner et al. (2004) used PCR to detect the presence of selected bacterial species in samples of acute periodontal abscesses collected from the United States and Brazil. They found that the prevalence of *P. intermedia, P. nigrescens, P. tannerae, F. nucleatum, P. gingivalis* markedly differed in two locations.

Apical periodontitis has a polymicrobial etiology and the functional role of species in this mixed endodontic consortium needs further efforts directed towards finding associations between them and clinical symptoms and conditions. It is claimed that, whether it is a failed endodontic treatment or a necrotic pulp space, the environment selects for microorganisms that possess traits suited to establishing and sustaining the disease process (Figdor and Sundqvist, 2007).

Conclusion

Findings indicated that the prevalence of some species found in the primary infection group were higher than in the secondary infection group. In this study there was a significant association between tenderness to percussion and *P. gingivalis*, pain with *P. melaninogenica* and swelling with both *P. gingivalis* and *P. melaninogenica*.

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