Presence of Oral Bacterial Species in Primary Endodontic Infections of Primary Teeth

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Objective: *Knowledge of the microbial composition of deciduous endodontic infections is limited. This study aimed to evaluate the presence of the 10 oral bacterial species in samples from primary tooth root canals by using microarray technology and to determine the association of these organisms with clinical conditions.* ***Study Design:*** *The samples were collected from 30 root canals of primary teeth with primer infection. The bacterial composition of the samples was semi-quantitatively defined using a microarray system (Parocheck®).* ***Results:*** *All the tested species were detected in the samples. Fusobacterium nucleatum was the most frequently isolated bacterium (96.7%), followed by Prevotella intermedia (86.7%), Parvimonas micra (83.3%), Treponema denticola (76.7%) and Tannerella forsythia (66.7%). These bacteria were also present in high levels. All pairs of bacterial species were positively associated (RR>1), except P.intermedia and P.micra. On average, five species (range:3-8) were detected per amplified sample. Root canals of teeth with >5 different species were statistically associated with periapical radiolucency (P=0.049).* ***Conclusions:*** *Primary teeth with endodontic infections show a highly diverse variety of bacteria, in which the most prevalent specie are present in high proportions. The well-directed use of the improved microarray technology will provide additional valuable information for causative factors associated with endodontic diseases, helping to develop more successful antibacterial or anti-inflammatory treatment strategies.*

Keywords: *endodontic infections, primary teeth, microarray analysis, oral bacteria.*

INTRODUCTION

Endodontic infections are symptomatic or asymptomatic inflammatory diseases caused by microbial infection of the root canal system.[1] Similar to most of the other human endogenous infections, a set of bacterial species usually organized in biofilm communities is involved with disease causation.[2] Several bacterial species, approximately equal proportions of Gram-positive and Gram-negative species, dominated by obligate anaerobes have been implicated in the etiology of endodontic infections in adults.[3-8] In contrast, few studies have assessed the microbial composition of deciduous endodontic infections,[9-14] utilizing different detection methods and focusing on a variable number of bacterial taxa. Knowledge of the endodontic infections of primary teeth is therefore far from complete.

Because of their anaerobic growth condition requirements, the identification of the oral bacteria using culturing techniques is time consumed, hardly applied and interpreted. New faster diagnostic methods with high sensitivity and specificity were qualified for detection of bacterial communities. PCR based DNA microarray systems are advanced computerized based techniques, which were developed for nucleotide sequence analysis with a width that could not be attained by any other technique with a single experiment. ParoCheck® microarray system (Greiner, Bio-one, Frickenhausen, Germany) is a diagnostic kit which can semi-quantitatively detect 10 or 20 oral bacteria at the same time. As it was originally developed to support the clinical diagnosis of chronic and aggressive forms of periodontitis, all of the species covered have also been described in endodontic infections.[15,16] So that, the usage of this technique may be useful for investigating the bacterial composition in endodontic infections of primary teeth.

The aim of the present study was to evaluate the presence of the 10 oral bacterial species in samples from primary tooth root canals by using microarray technology and to determine the association of these organisms with clinical conditions.

MATERIALS AND METHOD

Patient selection

Thirty children aged between 3-9 years (mean age, 6.50±1.55) attended to the Istanbul University Faculty of Dentistry, Department

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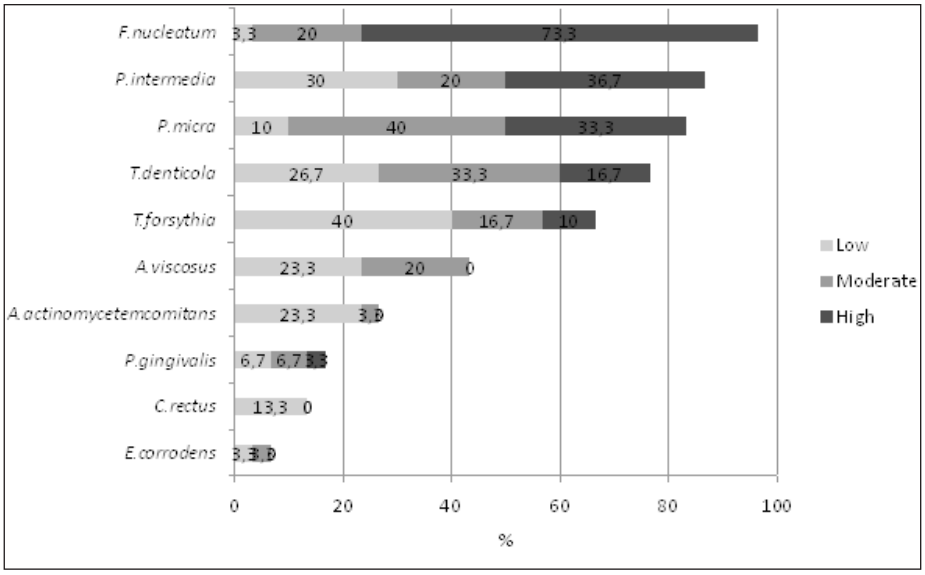

Figure 1. Stacked bar chart of frequency of detection and levels of bacterial species in root canal samples of teeth with primary endodontic infections (n=30). Total length of each bar stack indicates the percentage of positive samples, i.e., the prevalence of bacterial species. Different shades within each bar indicate the percentages of samples containing different levels of the species.

of Pediatric Dentistry and who had primary molar teeth in need of pulp therapy were included in this study. All clinical procedures were approved by Local Ethics Committee of the Istanbul University Faculty of Medicine (2012/726-1056) and informed consent was obtained from each parent. No study patient had received antibiotic treatment during the preceding 3 months or had a systemic disease.

Microbial samples were obtained from one root canal of 30 carious deciduous teeth which had intact roots or less than 2/3 of physiological root resorption with no previous root canal intervention was included in the study. Selected teeth showed negative results to electrical pulp testing (EPT), no significant gingival recession, were free of periodontal pockets more than 4 mm deep and had not received endodontic treatment, before. The sample was collected from only one tooth *per* patient. Age, gender, tooth type, internal status of the canal (dry canal, presence of hemorrhagic, clear, purulent exudates), and radiographic findings were recorded for each patient. The clinical and radiological investigations were done by one specialist (EB).

Microbiological sampling

The teeth were isolated with a rubber dam. The crown and the surrounding rubber dam were disinfected with 30% H_2O_2 (v/v) for 30 s followed by 2.5% NaOCl for an additional 30 s. Subsequently, 5% sodium thiosulphate was used to inactivate the disinfectant agents. An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile saline. Before entering the pulp chamber, the access cavity was disinfected following the same protocol described above.

All subsequent procedures were performed aseptically. The pulp chamber was accessed with sterile burs refrigerated by saline. Samples from multi-rooted teeth were taken from the largest root canal always associated with the periapical lesion. If the canals were dry, the samples were taken after introducing a small amount of sterile saline solution into the canal and then scraping the root canal walls with a size 15 K-type hand file (Dentsply Maillefer, Ballaigues, Switzerland). The samples were collected with two sterile paper points (ISO # 25 sized) that were consecutively placed into the main root canal to a total depth calculated from the preoperative radiograph, for 30 seconds. Afterwards, the paper points were pooled in a sterile empty tube and stored at -20ºC until use.

Microbiological analysis

For each sample, DNA was extracted and purified with the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich Co, St. Louis, USA) according to the manufacturer's instructions. The PCR amplification was carried out in a volume of 20 µl containing 18.8 µl Master Mix (delivered with the ParoCheck®s kit (Greiner Bio-One GmbH, Frickenhausen, Germany), containing dNTPs, and a forward as well as a 5' end Cy5-labelled reversed primer, universally targeting 16S rDNA, 1 µl sample DNA and 0.2 µl Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). The amplification was performed using a thermocycler (Eppendorf AG, Master Cycler Personel 22331, Hamburg, Germany) and the following temperature profile and cycles: pre-denaturation 94ºC for 1min; 45 cycles: 95ºC for 20 s, stringent annealing at 60ºC for 20 s, elongation 72ºC for 30 s; and final elongation after 45 cycles: 72ºC for 1min.

Hybridization on a chip was performed according to the instructions of the manufacturer (ParoCheck®, Greiner Bio-One GmbH). Each slide was incubated at 60ºC in a container with a saturated humid environment for at least 5 min. Afterwards, 30 µl of hybridization buffer and 5 µl of the target PCR product were mixed at room temperature, and 25 µl of this solution was transferred onto the slide surface and overlaid with a coverslip (25×25mm). The slide incubation was continued for an additional 10 min. The coverslip was removed and the slide was treated with a buffer system (supplied with the ParoCheck® kit) according to the manufacturer's instructions. The surface was dried by centrifugation. Results were automatically generated using a scanner (CheckScanner™ ,Greiner Bio-One GmbH) and processed by the ParoReport software (supplied with the ParoCheck® Kit, based on Gene Pixt, Axon Instruments Inc.). It

Table 1. Prevalence of microorganisms according to the clinical and radiographic signs in deciduous root canals

Bacterial species		n	Hemorrhagic exudate 14	Purulent exudate 12	Dry canal 11	Periapical radiolucency 21
E.corrodens	Positive	2	1	1	0	1
	Negative	28	13	11	11	20
C.recta	positive	4	2	1	2	3
	negative	26	12	11	9	18
P.gingivalis	positive	5	2	2	2	4
	negative	25	12	10	9	17
A.actinomycetemcomitans	positive	8	5	4	1	6
	negative	22	9	8	10	15
A.viscosus	positive	13	8	6	3	10
	negative	17	6	6	8	11
T.forsythia	positive	20	10	10	6	13
	negative	10	4	2	5	8
T.denticola	positive	23	12	9	9	18
	negative	7	2	3	2	3
P.micra	positive	25	11	10	10	19
	negative	5	3	3	1	2
P.intermedia	positive	26	14	12	7	18
	negative	4	0	0	4*	3
F.nucleatum	positive	29	14	12	10	21
	negative	1	0	0	1	0

**P.intermedia absence was associated with dry canals (P=0.012)*

interprets the control signals and restrains or releases the generation of an analysis-report with semi-quantitative labeling scheme. The results were estimated according to the labeling scheme: absent (-), low (+), moderate (++) and high (+++). The following 10 species can principally be identified by the ParoCheck10® microarray detection system: *Aggregatibacter actinomycetemcomitans, Actinomyces viscosus, Tannerella forsythia, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Parvimonas micra, Porphyromonas gingivalis, Prevotella intermedia* and *Treponema denticola*.

The sensitivity of all the PCR methods was evaluated using diluted DNA extracted from the reference strains (*A. actinomycetemcomitans* ATCC 33384, *F. nucleatum* ATCC 49256, *P. gingivalis* ATCC 33277 and *T. denticola* ATCC 35405 were used as positive controls and sterile distilled water as a negative control. Some of the positive cases were also analyzed with PCR using species specific 16SrRNA primers to verify the findings.[17]

Statistical analysis

Data collected for each sample were analyzed with NCSS 2007&PASS 2008 Statistical Software (Utah, USA). The prevalence of the target species was recorded as the percentage of the cases examined. The association of the species with exudate types and radiographic findings were determined. The Fisher's exact and Chi-square tests were used for comparison ($P< 0.05$). The Fisher's exact test was applied to test the null hypothesis that there was no relation between any of the most prevalent bacteria (bacteria which found in at least five samples) ($P < 0.05$). When there was a significant difference, indicating that a relationship between species was present, the odds ratio (OR) with a 95% confidence interval (CI) was calculated.

RESULTS

The number of sampled teeth with three, two and one root canals were 18 (60%), 11 (36.7%) and one (3.3%), respectively. Hemorrhagic and purulent exudates were detected in 14 (46.7%) and 12 (40%) root canals, while 11 (36.7%) were dry. No radiographic evidence of periapical radiolucency was obtained from nine (30%) of the teeth, while bifurcation, periapical, endo-perio+bifurcation and periapical+bifurcation lesions were obtained from 13 (43.3%), 3 (10%), 3 (10%) and 2 (6.7%) of the teeth, respectively. Table 1 shows the prevalence of microorganisms according to the clinical and radiographic symptoms in primary root canals.

All the tested species were detected in the samples. The number of different bacterial species detected in the samples ranged from 3 to 8 (mean, 5.2; median, 5). Root canals of teeth with more than six different species were statistically associated with periapical radiolucency. Table 2 shows the prevalence of the clinical and radiographic symptoms according to the species number (> 5 or ≤ 5 different species) detected per amplified sample. Periapical radiolucency was associated with high microbial diversity ($P= 0.049$).

The most prevalent bacterium was *F. nucleatum* corresponding to 29 of 30 cases (96.7%), which 22 of the positive cases (75.9 %) were with high levels. *P. intermedia, P .micra, T. denticola* and *T.*

Table 2. Prevalence of the clinical and radiographic signs according to the species number (> 5 or ≤ 5 different species) detected per amplified sample.

Signs	Number of bacteria per sample				
	> 5 (n=12)		≤ 5 (n=18)		
	n	%	n	%	P
Hemorrhagic exudate	7	58.3	7	38.9	0.457
Purulent exudate	4	33.3	8	44.4	0.709
Dry canal	3	25.0	8	44.4	0.442
Periapical radiolucency	11	91.7	10	55.6	0.049*

* Satistically significant

forsythia were detected from 26 (86.7%), 25 (83.3%), 23 (76.7%) and 20 (66.7%) of the cases, respectively. These bacteria were also present in high levels. Prevalence values for the 10 bacteria are depicted in Figure 1.

For *A. viscosus* and *A. actinomycetemcomitans* combination, the null hypothesis had to be rejected and therefore indicated a positive association (P: 0.005, OR: 20, 95% CI: 2-196). Contrariwise, *T. denticola* and *A. actinomycetemcomitans* combination indicated a negative association (P: 0.007, OR: 0.06, 95% CI: 0.01-0.46). Because of their paucity, ORs were not calculated for *C. recta* and *E. corrodens* (Table 3).

DISCUSSION

Root canal infections are polymicrobial and predominated by obligate and facultative anaerobes.[3] However, there are few studies concerning root canal microbiota of primary teeth. Marsh and Largent[9] reported alpha hemolytic streptococci as the predominant microorganisms whereas other studies[18,19] reported that the most prevalent microorganisms in root canals of primary teeth with necrotic pulp and periapical lesions were *Streptococcus salivarius.* Anaerobic microorganisms represented over 70% of the microbiota in root canals of primary molars that had been treated unsuccessfully.[10] Recently, molecular methods have been used to detect fastidious and difficult-to-cultivate bacterial species from the *Tannerella, Treponema, Prevotella*, and *Porphyromonas* genera, which may not or hardly be identified by conventional culture methods.[20-22]

The purpose of the present investigation was to evaluate the microbiota of endodontic infections in primary teeth, using a microarray technology based on the detection of the pathogen-specific 16S rRNA coding DNA. This technology was reported to have higher sensitivity than culture based techniques with a detection limit of 100-500 cells.[15] One of the advantages of this method is that it allows simultaneous detection of 10 bacterial species in several samples while exhibiting high specificity. Thus this system is less time and labor consuming than conventional PCR techniques.

Considering that only target species can be detected by microarray technology, it fails to detect the unexpected species. This figure might well be larger to include nontarget taxa and taxa at levels below the detection limits of the assay. For instance, the DNA extraction procedure as well as the PCR-based techniques might lead to biased retrieval of amplicons, discriminating against some bacterial species.[23]

The microarray used in this study was originally developed to detect periodontopathogens. Although some 'typical' endodontic species, such as *Porphyromonas endodontalis, Enterococcus faecalis, Pseudoramibacter alactolytis, Filifactor alocis*, or *Dialister pneumosintes* are not represented on the DNA-chip which was used in our study**,** these species are infrequently detected in root canals of primary teeth with primer endodontic infections.[13,14] So, we think that this system is convenient for endodontic infections of the primary teeth.

In present molecular study, the most prevalent bacterial species was *F. nucleatum*, present in 96.7% of the samples. It was followed by other obligate anaerobes, such as *P. intermedia* (86.7%), *P. micra* (83.3%), *T. denticola (*76.7%) and *T. forsythia* (66.7%). *C. rectus* (13.3%) and *E. corrodens* (6.7%) were least prevalent species. In a similar study, using checkerboard DNA-DNA hybridization for

Table 3. OR analysis of the association of specific microorganisms with other microorganisms

Bacterium	OR (%95 confidence interval)						
	P. intermedia	*P. micra*	*T. denticola*	*T. forsythia*	*A. viscosus*	*A. actinomycetemcomitans*	*P. gingivalis*
F. nucleatum	0.0	0.0	0.0	∞	∞	∞	∞
P. intermedia		1.8 (0.15-22.37)	0.0	0.6 (0.06-6.96)	2.6 (0.24-28.09)	1.1 (0.10- 12.47)	0.5 (0.04-6.66)
P. micra			0.8 (0.07-8.52)	0.0	0.1 (0.01-1.46)	0.2 (0.02- 1.28)	0.8 (0.07-8.73)
T. denticola				0.8 (0.12-4.77)	1.0 (0.19-5.66)	0.06 (0.01-0.46)*	∞
T. forsythia					1.2 (0.26-5.73)	4.8 (0.51- 46.49)	2.3 (0.22-23.32)
A. viscosus						19.83 (2.00- 196.39)*	0.3 (0.03-2.98)
A. actinomycetemcomitans							0.6 (0.06-6.80)

**Statistically significant (P<0.05 by Fisher Exact test)*

levels of 83 bacterial taxa, Tavares *et al* [14] detected *P. intermedia (*96.9%), *T. forsythia* (56.2%), *F. nucleatum* ssp. (40-50.0%) in 40 samples from the root canal system of deciduous teeth. These findings were in contrast with the results reported by Ruviere *et al* [12] and Ito *et al* [24] where *P. intermedia* and *T. forsythia* were detected in lower percentages. Using conventional PCR techniques, Cogulu *et al* [13] found that *T. denticola* (16%) and *Porphyromonas gingivalis* (16%) were the most prevalent species, while they did not found *F. nucleatum* and *P. intermedia* in primary root canals. In above studies, *P. micra* was detected in lower percentages than in our study. In a previous study, *P. micra* was found to be the prevalent organism detected in necrotic root canals by the same microarray technology, but it was not recovered at all the same samples by culture analysis.[15] The authors suggested that, some media and conditions might favor the growth of facultative anaerobes, which then might inhibit or mask the colonies of obligate anaerobes, like *P. micra*.

Endodontic infections have heterogeneous etiology, where multiple bacterial combinations can play a role in disease causation. Bacterial communities become important instead of one special bacterium. Considering and interpreting the pathogens individually in endodontal infections, is insufficient to clarify their etiologies. Identification of the community members can reveal the presence of some species or group of species that may be important for the causation of some forms of disease. It is reasonable to realize that different magnitudes of disease, based on intensity of signs and symptoms, may be related to the species composition of the community. Study of species richness and abundance in endodontic communities continues to be of utmost importance, as community behavior is obviously dependent on its diversity.[25]

Recently, intercellular communication among bacteria has been demonstrated that results in collective behavior to enhance adaptation and defense. The use of intraspecies and interspecies quorum sensing may greatly enhance the chances of bacterial survival or may allow bacteria to build communities in which division of labor would grant the community numerous new properties and benefits.[1] Strains of *P. gingivalis*, *F. nucleatum*, and *P. intermedia* were recently found to produce quorum sensing signal molecules.[26] It is entirely possible that other endodontic pathogens also produce autoinducers, and the possibility exists that they are involved in adaptability to root canal environment and coordinate activity resulting in enhanced pathogenicity.[1]

The bacterial diversity in any environment is underestimated when assessed by culture-based techniques.[6,27] Infected root canals yielded a maximum of 10–12 species when assessed by culture methods,[28] while this range raises to 42–51 species in studies using culture-independent molecular methods in adults.[4,7] Using whole-genomic probes, Ruviere *et al*[12] detected 34 bacterial taxa in root canal samples from primary teeth. The authors found, on average 7.47 bacterial species (range: 0–10) in teeth exhibiting irreversible pulpitis and 11.45 species (range: 0–27) in teeth with pulp necrosis and chronic apical periodontitis. Tavares *et al* [14] detected 19 species (range: 3–66) using using checkerboard DNA-DNA hybridization for levels of 83 bacterial taxa. It suggests that the difference in numbers may be due to the primary teeth harboring a less complex microbiota or that infection in adults may have been present longer, allowing time for greater diversity to develop. While our study is limited with 10 bacteria, on average, 5 species (range: 3-8) were detected per amplified sample. The results demonstrated that, teeth with periapical radiolucency contained higher bacterial diversity. These differences in species richness help explain the long-held concept that the endodontic treatment of teeth with large lesions has a lower success rate than treatment of teeth with small or no lesions.[29]

One important ecological factor that helps to determine the composition of the root canal microbiota includes bacterial interactions.[29] Bacterial combinations in root canals may be more pathogenic than individual strains.[16,17] Positive bacterial interactions enhance the survival capacity of the interacting bacteria and enable different species to coexist in habitats where neither could exist alone. Positive interactions can also result in enhanced pathogenicity due to additive or synergistic effects.[29] In the present study, a positive association was determined between *A. viscosus* and *A. actinomycetemcomitans*, while depicted for most of the bacteria. Table 3 shows the OR extremes ∞ and 0 for a number of cases due to the high prevalence of some organisms (e.g., *F. nucleatum*).

CONCLUSIONS

The present study shows that endodontic infections of the primary teeth are highly diverse, presenting, on average, five species per sample among the detected 10 bacteria, confirming the polymicrobial nature of these infections. The data also suggest that teeth with periapical lesions contain much more bacterial diversity and the most prevalent species also represents in high proportions.

This microarray system is convenient for endodontic infections, however, more studies analyzing a larger number of samples using standardized sampling methods are needed to foster a better understanding of the pathogenesis of apical periodontitis, as well as aid the design of more efficient endodontic treatments.

Undoubtedly, the well-directed use of these methods will provide additional valuable information regarding the identification and understanding of the causative factors associated with endodontic diseases, helping to develop more successful treatment strategies.

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