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Polymerase Chain Reaction-based Simultaneous Detection of Selected Bacterial Species Associated with Closed Periapical Lesions

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Abstract:
Purpose: The aim of this study was to identify microorganisms which might be present in closed periapical lesions and to determine their relationship to conventional clinical parameters.
Methods: In 11 patients, samples were taken with paper points from 14 teeth with periapical radiolucency, in seven cases two samples were taken from two different sites of the same tooth (n = 21). Clinical parameters were determined. The 16S rDNA of eleven bacterial species could be simultaneously detected via a modified polymerase chain reaction (PCR) based technique.
Results: All samples contained more than one bacterial species. Most frequently, *M. micros* was detected alone or in combination with *E. faecalis*, *P. aeruginosa*, *E. coli*, *F. nucleatum* or *S. sanguinis*. When apical palpation was positive, often *M. micros* and *F. nucleatum* were both present. Other clinical symptoms like tenderness to percussion were not associated with a particular microflora.
Conclusions: In closed periapical lesions detected by radiography, specific bacteria were identified by means of a modified PCR technique. No clear associations between clinical symptoms and these bacteria were found. Surgical intervention might be indicated for some of the persistent lesions.

Key words: closed periapical lesion, PCR, pain pathology, root resection

Introduction

Therapy-resistant chronic periapical inflammation is still an area of considerable interest. Since the early 20th century, the question to which extent a periapical lesion can be considered as sterile, was an issue of debate for various groups of researchers. It was controversially discussed whether a periapical lesion is completely free from or is sparsely populated by bacteria [2, 16].

The number of bacteria present and the detection of the various bacterial species were dependent on the different identification methods. Grossman [14] detected microorganisms in 14.7% of all periapical lesions. The improvement of cultivating techniques for aerobic and anaerobic species resulted in an increasing detection of periapical bacteria [9, 32]. By means of histochemical analysis, Winkler et al. [34] detected microorganisms in the periapical area in 96.6% of all surgically treated endodontic cases. In a more recent study, various bacteria could be directly visualized in periapical lesions of asymptomatic root-filled teeth, using fluorescence in situ hybridization [31]. Nowadays, PCR is an elegant method for the identification of microorganisms, which makes cultivation unnecessary. The use of modern molecular techniques has lead to a better understanding of endodontic infections, including the detection of bacteria in periapical lesions [33].

In the present study we evaluated samples from patients with so-called "closed periapical lesions". A closed periapical lesion describes lesions on teeth with pulpal or periapical disease without any direct communication with the oral environment and associated microorganisms. They are usually dominated by opportunistic oral microorganisms that originate from caries lesions, tooth fracture or crack-lines, leakage around restorations, poor-quality root canal treatment [5, 6], periodontal defects (infrabony pockets communicating with the apex), sinus tracts, and communication with the maxillary sinus [1, 17]. While some authors [23] have occasionally detected *Candida* sp., the predominant microorganisms that have been isolated from such lesions are various bacterial species such as *Actinomyces* spp., *Propionibacterium* spp., *Streptococcus* spp., *Staphylococcus* spp., *Porphyromonas gingivalis*, *Micromonas micros* (formerly *Peptostreptococcus micros*) and Gram-negative enterics [1] or *Prevotella intermedia*, *Bifidobacterium* spp., *Fusobacterium nucleatum* [20], *Streptococcus sanguinis*, the *Streptococcus milleri*-group and *Bacteroides* spp. [8, 27]. Hong et al.[15] reported that lipopolysaccharide from *F. nucleatum* can play an important role in bone resorption associated with periapical lesions.

The purpose of this study was the simultaneous identification of bacterial species implicated in periapical disease, isolated from closed periapical lesions, using a modified polymerase chain reaction (PCR) method, and to determine their relationship to the conventional clinical parameters.

Material and Methods

The study group comprised 11 patients and 14 teeth with the diagnosis "chronic periapical periodontitis." The clinical assessment, diagnosis and therapy were performed by the same oral surgeon. The radiographs (orthopantomograms or dental X-rays) showed a periapical radiolucency (osteolysis) sized one to six millimeters (e.g. Fig. 1).

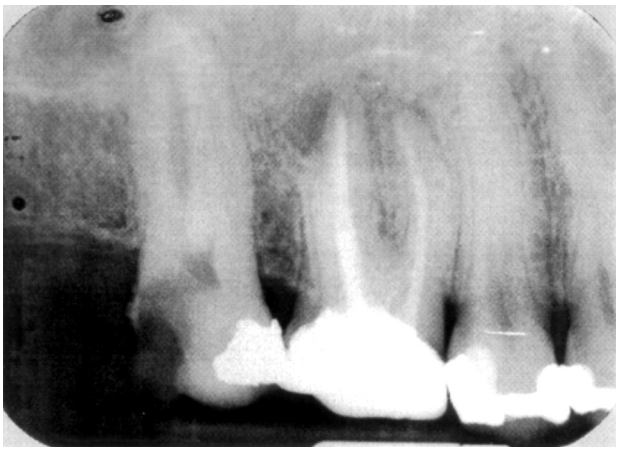

Fig. 1. Radiograph of a patient with periapical lesion.

The pocket depths measured at four reading points (mesiolabial, mesiopalatinal, distolabial, distopalatinal), were <3 mm. The clinical status of the anatomical crowns was in all cases rated as sufficient: no caries lesions, fractures or crack-lines or leakage around restorations were present. An apical pressure point could be ascertained in 8 of the 14 cases, in four cases tenderness to percussion was detectable (pain pathology).

The status and quality of the root canal fillings, which had been placed at least 1 but no more than 10 years ago, was evaluated by radiography; in 13 of the 14 teeth the fillings were rated as good, in one tooth the filling was considered as poor. Both ratings were done according to the criteria of Ray and Trope [26]. In 7 cases a histological evaluation of the periapical tissue, taken during root resection, was carried out.

Before oral surgery took place, all patients rinsed their mouth with a 2% solution of chlorhexidine digluconate for one minute. After the formation of a mucoperiosteum flap and osteotomy but before root resection, sampling took place. The samples were taken with sterile paper points (Roeko, Langenau, Germany, size ISO25), 14 from the inside of the periapical lesion. The paper points were inserted and left in place for two seconds. In seven cases a second sample was taken at the apex of the tooth before surgical resection. Aseptic techniques were used during surgical procedures and cooling was performed with a sterile sodium chloride solution. All samples were transferred to the lab and immediately processed for PCR analysis.

In order to isolate the genomic DNA from all bacterial species present, the paper points were shaken in one ml of solution I, containing tris (hydroxymethyl) amino methane, for about 60 seconds, and the total DNA was isolated according to the standard protocol for bacteria (NucleoSpin tissue-kit, Macherey-Nagel, Düren, Germany). The DNA was purified using an ethanol precipitation step: 2 µl pellet paint® co-precipitant (Novagen, Merck Biosciences, Darmstadt, Germany) and 20 µl sodium acetate (3M) were pipetted to the DNA, 400 µl ethanol was added and the solution was cooled to and kept at -20 °C for 12 hours. 40 PCR cycles amplified a fragment of the bacterial 16S rDNA of about 700 base pairs from the DNA mixture using a pair of universal primers (Thermo Electron, Ulm, Germany) (Table 1) and five U/µl Taq polymerase (peQLab, Erlangen, Germany), at a temperature of 55 °C. The PCR products of the 16S rDNA of all bacteria in the sample were used as templates in a second nested PCR. In another 40 PCR cycles with primers [11] (Table 1) (Thermo Electron) that bind specifically to the 16S rDNA fragments of the eleven bacterial species listed below, specific segments of these amplicons were synthesized. The resulting fragments were separated according to size by means of 3% (low melting point) agarose gel electrophoresis and visualized with ethidium bromide under UV light, and then used for the identification of the bacteria. The detection limit was 50 bacterial cells as determined with *E. coli* K12. The specificity of the nested PCR was verified by their fragment size, commercially available strains of the respective bacterial species (DSMZ, Braunschweig, Germany) served as controls in each case. During this study the following eleven microorganisms were investigated: Gram-positive facultative anaerobic cocci: *Streptococcus mitis* ATCC 49456^T, *Streptococcus oralis* ATCC 35037^T, *Streptococcus sanguinis* ATCC 10556^T; *Staphylococcus aureus* ATCC 12600^T, and *Enterococcus faecalis* ATCC 19433^T; Gram-positive anaerobic cocci: *Micromonas micros* (formerly *Peptostreptococcus micros*) ATCC 33270^T; Gram-negative anaerobic rods: *Fusobacterium nucleatum* ssp. *polymorphum* ATCC 10953^T, *Porphyromonas gingivalis* ATCC 33277^T; Gram-positive facultative anaerobic rods: *Lactobacillus casei* ATCC 393^T; Gram-negative facultative anaerobic rods: *Pseudomonas aeruginosa* ATCC 10145^T, and *Escherichia coli* ATCC 11775^T.

P. gingivalis and *S. oralis* served as controls in order to exclude a possible contamination from the sulcus or the mucous membranes during sampling.

A negative control, containing sterile aqua dest. instead of a sample was included in order to exclude a possible contamination from the environment during PCR.

Methodical errors during sampling were eliminated by duplicate testing.

Results

The bacterial species found in samples from patients with pocket depths <3 mm and closed periapical lesions can be summarized as follows (Table 2): in almost all cases we found *M. micros*, typically in combination with up to six other species, for example with *F. nucleatum*, *E. faecalis*, *S. sanguinis*, *E. coli* or *P.*

Table 1. Primer design for the identification of bacterial species via nested PCR (annealing temperature 55°C)

	up	**down**
universal	GCGVACGGGTGAGTAAC Tm 60.5 °C	GGACTACCAGGGTATCTAATCC Tm 61.6 °C
S. aureus	AACCGCATGGTTCAAAAG Tm 61 °C	TGTGCACAGTTACTTACACATATG Tm 61 °C
F. nucleatum	CCTADGATTATGAAAGCTATATGC Tm 60.8 °C	CTTCTGTTGGTACCGTCATT Tm 59.5 °C
L. casei	ACCGCATGGTTCTTGGCTG Tm 61 °C	GGTTGGATACCGTCACGC Tm 63.4 °C
E. faecalis	TGGCATAAGAGTGAAAGGC Tm 60.6 °C	TGGTTAGATACCGTCAGGG Tm 60.0 °C
P. aeruginosa	CCGCTAATACCGCATACG Tm 62.4 °C	GTAACGTCAAACAGCAAGGTA Tm 60.5 °C
S. mitis	GTAGATGTTGCATGACATTTGG Tm 62.9 °C	GTGAACTTTCCACTCTCACACT Tm 60.4 °C
M. micros	GGTCAAAGATTTATCGGTGTA Tm 59.5 °C	CCTCTCAGACCGGCWACTG Tm 62.3 °C
S. oralis	GTAGATGTTGCATGACATTTGA Tm 61.6 °C	GTGAACTTTCCACTCTCACAC Tm 47.4 °C
E. coli	CGGTAGCTAATACCTCATAACG Tm 60.8 °C	ATGAGCAAAGGTATTAACTTTACT Tm 59.9 °C
S. sanguinis	ATAGCTNATACCGCATAAAATTG Tm 61.8 °C	GTAGATGTTGCATGACATTTGS Tm 47.7 ° C
P. gingivalis	TATTATTGCATGATATTACAAGGA Tm 60.2 °C	ATTCTTACGGTACATTCAATGC Tm 61.6 °C

Tm: Melting temperature

Table 2. Bacterial species found in samples from patients with pocket depth < 3 mm.

Patient (tooth, sample)	*M. micros*	*S. aureus*	*E. coli*	*F. nucleatum*	*S. mitis*	*P. aeruginosa*	*S. sanguinis*	*P. gingivalis*	*L. casei*	*E. faecalis*
1 (36a)	X	X								
1 (36b)	X									
2 (24)		X	X	X						
2 (25)		X	X	X						
3 (25)	X	X	X	X	X					
4 (15)	X			X		X				
5 (13)	X		X	X		X				
6 (26)	X		X	X		X				
7 (36a)	X		X	X		X	X			X
7 (36b)	X		X	X		X	X			X
7 (37a)	X		X	X		X				X
7 (37b)	X		X			X				X
8 (32)	X					X	X			X
9 (16a)	X		X			X	X	X	X	X
9 (16b)	X		X	X		X	X		X	X
9 (11a)	X		X			X	X		X	X
9 (11b)	X					X	X		X	X
10 (21a)	X		X			X	X			X
10 (21b)	X		X			X				X
11 (25a)	X					X	X		X	X
11 (25b)	X					X	X		X	X

Table 3. Pain pathology and other clinical parameters in patients with pocket depth < 3 mm.

	apical palpation positive n = 8	apical palpation negative n = 6	percussion testing positive n = 4	percussion testing negative n = 10	apical granuloma n = 4	cysts n = 3
Micromonas micros	8	4	4	8	4	2
Pseudomonas aeruginosa	6	4	3	7	3	2
Escherichia coli	4	4	2	7	1	2
Fusobacterium nucleatum	6	2	3	6	2	1
Enterococcus faecalis	3	4	1	6	1	2
Streptococcus sanguinis	2	4	1	5	1	2
Lactobacillus casei	0	3	0	3	1	0
Streptococcus mitis	1	0	1	0	0	0
Staphylococcus aureus	2	2	1	3	1	1
Streptococcus oralis	0	0	0	0	0	0
Porphyromonas gingivalis	0	1	0	1	0	0

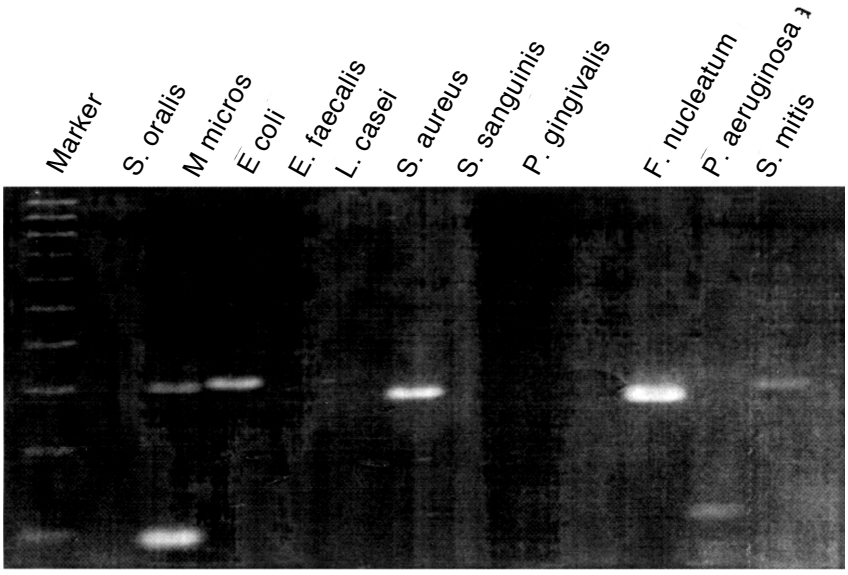

Fig. 2. Gel electrophoresis of a sample taken from the center of a granuloma.

aeruginosa (e.g. Fig. 2). Where two samples were taken, the bacterial findings were in good agreement. In most cases, they were either identical or differed with respect to only one species.

There was no correlation between the histological diagnosis 'cyst' (n = 3) or 'apical granuloma' (n = 4) and the bacterial findings. *Porphyromonas gingivalis* was found only in one case, *S. oralis* was never detected.

In Table 3 the correlation of pain pathology to the detected bacteria is listed. In 6 of 8 cases, the clinical parameter positive apical palpation was correlated with the occurrence of *M. micros* together with *F. nucleatum*. This was also observed in 3 of the 4 cases where percussion testing was positive, whereas in the periapical lesions from the ten teeth, where percussion testing was negative, in the majority of the cases only one or the other organism was detected. In contrast, *L. casei* was only identified in cases where apical palpation or percussion testing were negative. It was not possible to correlate the other detected bacteria or bacterial combinations with pain pathology.

Discussion

The results of the microbiological analysis of the samples taken from closed periapical lesions in patients with pocket depths of <3mm, strongly support the existence of polymicrobial infections, as has been shown for the composition of the pathogenic flora in root canals, periapical lesions or pre- and postoperative apicoectomy sites [8, 20, 27, 28]. This confirms that endodontically treated teeth cannot be considered to be sterile, and is in good agreement with investigations of other working groups [4, 7, 18, 19]. However, a statement on the proportions of the individual species in the samples cannot be given, since the PCR technique allows only the qualitative determination of the microbial composition. The detection of *E. faecalis*, a facultative anaerobic species mostly found in failures of en-

dodontically treated teeth, can be attributed to the fact that this organism can infect the periapical region via the root canal [24].

In accordance with Molander et al. [18] we found that no correlation could be established between the identified bacteria and clinical parameters like the status and quality of the root canal fillings or the size of the periapical lesion that had been evaluated by radiographs.

Likewise, the results from histological evaluations, giving rise to the diagnosis 'cyst' or 'granuloma', could not be associated with one or more particular bacterial species.

Not all radiographically diagnosed periapical lesions (see Fig. 1) necessarily contain bacterial species that are typically associated with infected root canals. Scarred healing of periapical infectious diseases, reaction to foreign bodies and periapical cysts can also cause an osteolysis with the same radiographic finding [21, 22, 29].

Gomes et al. correlated the occurrence of *Prevotella* ssp. as well as *Peptostreptococcus* ssp. with pain pathology [12, 13] and *Peptostreptococcus* and *Fusobacterium* with tenderness to percussion [13]. In the present study, neither typical combinations of bacteria in polymicrobial infections, nor the presence of any single species like e.g. *Micromonas micros*, a typical organism present in periapical inflammation, clearly correlated with the pain pathology. While Siqueira et al. reported [30] that *Micromonas micros* had only an association of 25% with "tenderness to percussion", in this study *M. micros* was detected in all teeth with these clinical symptoms, but it was also found in the majority of the asymptomatic cases.

In the present study *P. aeruginosa* was found in a high number of cases. This is not an organism that belongs to the normal oral microflora. Instead it is an opportunistic pathogen which is often isolated from the environment, but is also capable of colonizing various sites of the human body. It can even interact with saliva and adhere to buccal epithelial cells, and can sometimes establish itself in the oral cavity. Several authors [3, 10, 25] have found *P. aeruginosa* in the root canal or in periapical lesions. Other authors argue that the presence of *P. aeruginosa* is a sign for contamination from the environment. While we can exclude contamination of the samples from the sulcus and the oral mucosa, since *P. gingivalis* and *S. oralis*, typical colonizers of these sites, were only rarely found, a contamination with *P. aeruginosa* from the environment at some point during surgery is still possible. It can also not be excluded that dead organisms, remaining behind after rinsing with the 2% chlorhexidine solution were detected, since the DNA from such organisms is also multiplied by the Taq polymerase. Cheung et al. [10] argue that due to a poor standard of asepsis *P. aeruginosa* could have been introduced into the root canal during previous endodontic treatments. This is supported by the fact that *P. aeruginosa* has only been reported in samples from root canal treated teeth [25].

Based on the results of this study the following conclusions can be drawn: closed apical lesions at teeth with treated root canals cannot be considered to be sterile. They are likely to be infected, which was shown by the simultaneous detection of various bacteria using a modified PCR technique [11]. A more detailed knowledge of the microflora from such lesions can be useful for choosing the appropriate treatment strategies or the materials for retrograde root-canal fillings. Antibacterial properties of such materials might help prevent recurrence of periapical lesions. Surgical intervention might be indicated, if these lesions persist.

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