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Anaerobic microorganisms in root canals of human teeth with chronic apical periodontitis detected by indirect immunofluorescence

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Abstract – Aiming to assess the presence of selected anaerobic microorganisms in root canals of human teeth with chronic apical periodontitis, 25 central and lateral upper incisors presenting with radiographic evidence of chronic apical periodontitis were studied. The pulp chamber was opened under aseptic conditions and samples of the root canal content were collected with sterile absorbent paper points, which were placed and dispersed in test tubes containing reduced transport medium (RTF). Aliquots were dried on glass slides and stained by indirect immunofluorescence for detection of *Actinomyces viscosus*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia*. The results showed a positive indirect immunofluorescence reaction in 24 of the 25 samples. Fourteen were positive for the specie *Actinomyces viscosus*, 12 for *Prevotella intermedia*, 10 for *Fusobacterium nucleatum* and 4 for *Porphyromonas gingivalis*. A semiquantitative assay was easily implemented for assessment of degree of infection by the organisms in individual cases.

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Key words: apical periodontitis; indirect immunofluorescence; anaerobic microorganisms.

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The important role of the bacteria in the pathogenesis of the pulpal and periapical disease has been extensively demonstrated (1–4), and the microorganisms present in the root canal can survive and maintain a periapical infectious process (5). Therefore, one of the prime objectives of endodontic treatment is the elimination of infection from the root canal system and the prevention of a reinfection (6, 7).

Until the 1970s the most commonly identified bacteria in infected root canals were the aerobes and the facultative anaerobes. When strictly anaerobic culture techniques are used, it has been shown that, the microorganisms which predominated in the infected root canal system of teeth with chronic apical periodontitis are anaerobes.

Besides the culture techniques, immunocytochemical methods demonstrate the presence of anaerobic microorganisms. Particularly, indirect immunofluorescence is a rapid method which may be used to detect live and dead microorganisms (8–10).

Thus, our aim was to assess the presence of *Actinomyces viscosus*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in human teeth with chronic apical periodontitis using the indirect immunofluorescence technique in material sampled before the biomechanical preparation.

Material and methods

Twenty-five upper lateral and central incisor teeth with caries lesions, pulp necrosis, and radiographically demonstrable, chronic apical periodontitis were selected from 23 adult 20- to 30-year-old patients.

After isolation with a rubber dam the operative field was disinfected with 0.3% iodoethanol.

When coronary access and pulp chamber irrigation with sterile physiological saline had been performed, the sampling for the bacteriological examination was effected. Sterile paper points were introduced for approximately one minute in the root canal, in increasing sizes from 15 to 30. The paper points were removed and placed in a 13×100 mm test tube containing 2 ml of reduced transport fluid (RTF), prepared according to Syed & Loesche (11). In this sampling as many paper points as necessary were utilized successively until an apparently dry paper point could be drawn from the root canal (3 to 4 paper points). Subsequent treatment procedures were performed according to conventional methods using chemomechanical preparation and antibacterial dressing with calcium hydroxide paste, and the root canal were filled using the lateral condensation technique with gutta-percha points and root canal sealer (Sealapex, Sybron-Kerr, São Paulo, Brazil).

Indirect immunofluorescence

Sterile barbed wings were placed inside the test tubes containing the samples and stirring in a shaker (Mixtron-Toptronix) was done in order to effect the desorption of the microorganisms from the paper points.

Indirect immunofluorescence assays were performed according to Van Poperin & Lopatin (12) and Zambon et al. (13). Briefly, a 10 µl aliquot of each sample was placed in one of the well of special glass slides (Lioserum, SP, BZ) for the immunological reaction. In one of the wells, 10 µl of an *Escherichia coli* suspension were applied for a negative control and a pooled suspension containg *Actinomyces viscosus*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis* (provided by D. E. Lopatin, University of Michigan School of Dentistry, Ann Harbor, Michigan, USA) was applied to another well as a positive control.

After drying at room temperature, the glass slides were submitted to gentle flame fixation. Ten µl of the specific antibodies: anti-*Actinomyces viscosus*, *Fusobacterium nucleatum*, *Prevotella intermedia* or *Porphyromonas gingivalis* (also provided by D. E. Lopatin) appropriately diluted in phosphate buffered saline solution, pH 7.2–0.15 M with 2.0% bovine albumin serum-AB (PBS-2.0-AB) were applied and incubated during 10 minutes at 37°C in a humid chamber. After incubation, the glass slides were washed with phosphate buffered saline containing 0.05% Tween 20 (PBS-T) for 10 minutes, then washed with distilled water and dried at room temperature. Afterwards, 10 µl of fluorescein-conjugated, goat anti-rabbit gammaglobulin (Sigma F9887) diluted in PBS-2.0-AB were applied to each of the wells and incubated during 10 minutes at 37°C in a wet chamber, then washed as described above. After drying, the glass slides were mounted with 0.05 ml buffered glycerol-PBS (2:1, v/v, pH 9.0) with 0.05% Trypan blue (Merck), covered with coverglasses fixed with nail varnish, placed in a humid chamber and analyzed within two hours in a fluorescence microscope JENAMED 2, Carl Zeiss, GF PW 10×, Planachromat HI 100×.

The samples were examined for the presence of fluorescent cells. A total of 100 bacterial cells, or the whole smear, were counted in each well.

The results were registered as number of + signs: ++++, when more than 10 cells were present in most of the fields; +++, when 5–10 were observed in most of the fields; ++ from 1 to 4 and +, when up to 10 cells were observed all over the smear. The number of + signs was considered "infection grade" of "infection intensity".

Results

Table 1 shows the presence of four species of bacteria (*Actinomyces viscosus*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis*) in the sampled root

Table 1. Presence of species of anaerobes (*A. viscosus*, *P. intermedia*, *F. nucleatum* and *P. gingivalis*) and grade of infection in the samples collected in root canals before biomechanics (immunofluorescence technique).

	Species of anaerobes			
Cases	*A. viscosus*	*P. intermedia*	*F. nucleatum*	*P. gingivalis*
P1	++++	++++	–	–
P2	–	–	+	–
P3	+	+++	–	–
P4	+	–	++++	–
P5	++++	+	–	–
P6	+	–	–	–
P7	–	–	++	–
P8	++++	–	++++	–
P9	+	+	–	+
P10	–	–	+	–
P11	++++	++	+	–
P12	–	–	++	–
P13	+	+	++	–
P14	–	–	–	+
P15	–	+	–	–
P16	+	++	–	–
P17	–	–	–	–
P18	+	–	–	+
P19	–	–	+++	–
P20	–	–	–	+
P21	+	+	–	–
P22	+	++++	–	–
P23	–	++++	–	–
P24	–	++++	++++	–
P25	+	–	–	–

The number of signs indicates the intensity of the infection: ++++ (more than 10 cells in most of the fields examined), +++ (from 5 to 10 cells in most of the fields), ++ (from 1 to 4 cells in most of the fields), + (up to 10 cells all over the smear), – (non-detected bacterium).

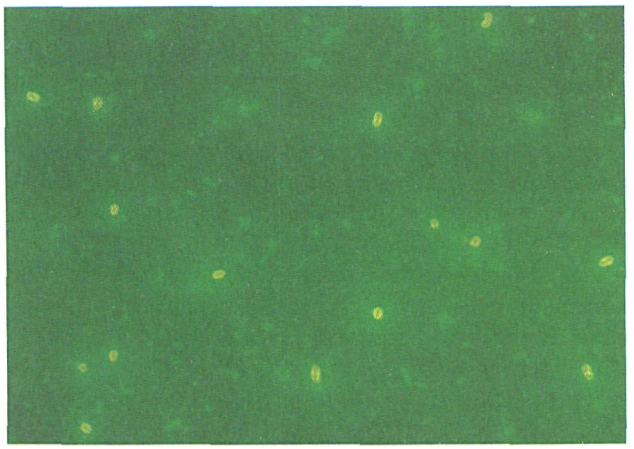

Fig. 1. Indirect immunofluorescence showing *Prevotella intermedia* (case #24).

canals. Twenty-four samples were positive for at least one of the searched species: 11 samples showed only one species of anaerobes; and 13 samples harbored more than one species of the latter 10 harbored two species and 3 harbored three species. In none of the 24 positive samples the simultanous presence of the four investigated species was observed.

For every species of bacteria the intensity of the infection varied among the samples, with the exception of *Porphyromonas gingivalis* which showed only a grade 1 positive immunofluorescence reaction.

Discussion

Using the indirect immunofluorescence technique in the material sampled in 25 root canals before biomechanical preparation, the specie *Actinomyces viscosus* was detected in 56% of cases. This result is similar to the 60.0% observed by Gohean et al. (10) in samples collected in root canals of teeth with periapical lesions submitted to the indirect immunofluorescence reaction. The presence of *Actinomyces* was also demonstrated by Borssén & Sundqvist (14) using the indirect immunofluorescence in 25 (10.6%) strains of the 235 positive cultures.

Actinomyces has a known pathogenic potential (9, 14, 15). It may survive in the periapical tissue and may jeopardize the normal process of repair and may be the cause of periapical lesions resistant to the endodontic treatment (5, 7, 16).

The prevalence of *Prevotella intermedia* (48.0%) observed in this study agreed with the observations of Pantera Jr. et al. (8), who detected *B. intermedius* (*Prevotella intermedia*) in 43.0% of the root canal samples tested with indirect immunofluorescence. The presence of *B. intermedius* (*Prevotella intermedia*) was also observed by immunofluorescence in the periapical granuloma by Barnett et al. (9).

Also, the prevalence of *Porphyromonas gingivalis* detected in this study in 16.0% of the cases was similar to the observed by Pantera Jr. et al. (8) who detected *B. gingivalis* (*Porphyromonas gingivalis*) in 15.0% of the samples from human teeth with periapical lesions.

The *Fusobacterium nucleatum* prevalence of 40.0% cannot be directly compared to other findings as there are no other studies using indirect immunofluorescence to detect this microorganism. However, Byström & Sundqvist (17), Wayman et al. (18) and Sundqvist (15), among many others have reported the isolation of *Fusobacterium nucleatum* in root canals of human teeth with necrotic pulp.

In a comparison of the anaerobic culture method with the indirect immunofluorescence reaction, Pantera Jr. et al. (8) showed the presence of *Bacteroides* species in 27.0% of the bacteriological samples from extracted teeth and in 20.0% taken from patients, while using the indirect immunofluorescence technique species *Bacteroides* were detected in 86.0 and 40.9% of the same samples, respectively. *Bacteroides intermedius* (*Prevotella intermedia*) was observed, by the culture method, in only 11.0% of the samples collected in patients; however, it was identified in 43.0% of these samples by the indirect immunofluorescence reaction. *Bacteroides gingivalis* (*Porphyromonas gingivalis*) isolated in 4.0% of the samples from patients through indirect immunofluorescence was found in 15.0% of these samples. More recently, Yi et al. (19) used the culture technique and the indirect immunofluorescence with monoclonal antibody to detect *P. endodontalis* in 24 samples of pus from root canals of teeth with periapical abscess. They observed that the culture was positive in 25% of the cases, while indirect immunofluorescence was positive in 37.5%. Thus, they concluded that the immunofluorescence technique is less time-consuming and more sensitive to detect *P. endodontalis*, dispensing the bacterial culture.

These data demonstrated the importance of the indirect immunofluorescence reaction when compared to the microbiological culture method.

The value of the indirect immunofluorescence reaction was also reported by Loesche et al. (20), when the anaerobic culture technique, the DNA probing, the ELISA reaction and the indirect immunofluorescence were compared in the detection of periodontopathogenic microorganisms.

For indirect immunofluorescence assay it is important the use of a adequate dilution of antisera, both bacteria specific as well as fluosrescein-conjugate gammaglobulin. The use-dilution of each antibody were determined by the "block titletion" technique as proposed by Bier (21).

Our results allowed us to confirm that the indirect immunofluorescence was an effective, quick and prac-

tical method to detect anaerobic microorganisms in root canals.

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