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Identification of *Synergistetes* in endodontic infections

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ABSTRACT

The bacterial phylum *Synergistetes* consists of Gram-negative anaerobes. Oral *Synergistetes* are divided in two main clusters, namely A and B. Increasing evidence demonstrates their involvement in etiology of oral infections, including apical periodontitis. This condition causes bone loss around the apex of the tooth, subsequent to pulp inflammation (pulpitis). Although the presence of *Synergistetes* has been confirmed in endodontic infections by molecular methods, these have not been morphologically identified in the affected apical region, and their prevalence among different endodontic infections has not been determined. Therefore, the aim of this study was to evaluate the prevalence, levels and morphology of oral *Synergistetes* clusters A and B, in apical root canal samples obtained of teeth with irreversible pulpitis, pulp necrosis and apical periodontitis, or previously root-filled teeth with apical periodontitis. For their detection, fluorescence *in situ* hybridization and epifluorescence microscopy were used. *Synergistetes* cluster A was not detected in pulpitis, but was found in both apical periodontitis groups, more frequently and at higher ranges in teeth which were previously root filled. Microscopically, they appeared as straight or slightly curved long rods. *Synergistetes* cluster B was not detected in any of the cases. *Fusobacteria* and *Actinomyces*, which are well-established taxa in endodontic infections, were detected more frequently and at higher ranges than *Synergistetes*. In conclusion, *Synergistetes* cluster A constitutes part of the mixed apical microbiota in apical periodontitis, and may be involved in its pathogenesis.

1. Introduction

Synergistetes is a recently identified bacterial phylum consisting of Gram-negative anaerobes. They are found in several microenvironments and constitute part of the human microbiota in health and disease [1–4], including the oral microbiota [5]. Phylogenetically, the oral *Synergistetes* are divided principally into clusters, namely cluster A and cluster B [2,3]. Considerable work on the role of *Synergistetes* has been made in the field of periodontal infections. They are detected more frequently and more abundantly in subgingival plaque from periodontitis-affected than healthy sites [2], or in the saliva of patients with periodontitis, compared to healthy individuals [6]. Accordingly, dental plaque from periodontitis-affected sites exhibits higher clonal abundance and diversity of *Synergistetes*, in comparison to healthy sites [7]. The presence of *Synergistetes* in dental plaque is also more pronounced in necrotizing ulcerative gingivitis, compared to plaque-induced gingivitis [8].

Widespread oral infections, such as caries, periodontitis, and apical periodontitis, are of largely opportunistic nature [9]. Apical periodontitis (AP) is the outcome of endodontic infection. It is very prevalent among adults, with an estimated one-third of the population being affected [10]. The initial steps of the disease involve the microbial invasion and inflammation of the pulpal tissue (pulpitis), primarily as a result of dental caries [11,12]. Persistent inflammation inside the root canal system causes degradation of the pulpal tissue [13] and allows for the progression of the endodontic infection. Histopathologically, this leads to the establishment of an inflammatory lesion in the bone around the apical region of the tooth, which is characteristic of AP, aiming to keep under control the recurring the infection in the root canal system [14].

Synergistetes have been frequently detected at elevated numbers in root canals of teeth with chronic endodontic infections, such as AP, as identified by polymerase chain reaction (PCR)-based

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methods and sequencing analyses [15,16]. The size of the total *Synergistes* population in necrotic teeth with AP is reported to range between 10^4 and 10^6 16S rRNA gene copies, while their proportion constitutes <1.0% of the total microbial community, allegedly within the detection range of other pathogens characteristic of endodontic infections [17]. Yet the differential presence of *Synergistetes* clusters A and B in the apical region of teeth with various clinical endodontic diagnoses has not been determined. Hence, the aim of this study was to evaluate the prevalence, levels and morphology of oral *Synergistetes* clusters A and B, in apical samples obtained of teeth with irreversible pulpitis but normal apical tissues, pulp necrotic teeth with AP, or previously root-filled teeth with AP. Comparatively, the presence of *Fusobacteria* and *Actinomyces* was also evaluated, as these are well-established taxa in mixed endodontic infections.

2. Materials and methods

2.1. Patients, procedures and sample collection

This study was approved by the Ethics Review Board of the Canton of Zürich (KEK-ZH-No. 2011-0253/4) and was conducted in accordance with the guidelines of the World Medical Association Declaration of Helsinki. The participating patients were in need of root canal treatment, and were treated at the Clinic for Preventive Dentistry, Periodontology and Cardiology, University of Zürich, Center of Dental Medicine, Switzerland by one operator specialized in endodontics (DKR). The patients were asked if they were willing to participate in the study when they were of full age (≥18 y), and were excluded from the study if they: (i) were unwilling to participate, (ii) were under long-term anti-inflammatory medication, such as immunosuppressive chemotherapy or any antibiotic medication, (iii) suffered from systemic illness (i.e. cardiovascular and respiratory disease, diabetes mellitus, HIV infection or hepatitis), or (iv) were pregnant or in lactation. The clinical condition was diagnosed according to the patient's case history, clinical inspection, palpation, tenderness to percussion, vitality testing, probing depth and single-tooth radiographic examination. The clinical conditions included in the study were irreversible pulpitis but normal apical tissues (pulpitis, $n = 27$), pulp necrotic teeth with apical periodontitis (N-AP, $n = 33$), or apical periodontitis associated with a root-filled tooth (R-AP, $n = 21$). Only one tooth per patient was included in the study. None of the sampled teeth exhibited a confirmed combined endodontic-periodontal lesion. All patients who entered the study gave written informed consent. All operative procedures were performed under a dental microscope and rubber dam isolation. The endodontic access was prepared with a sterile diamond-coated bur and the root canals were instrumented using ProTaper instruments (Maillefer Dentsply, Ballaigues, Switzerland), while endodontic working length was determined endometrically (Root ZX mini, J Morita Corp., Tustin, CA) with a hand file (Maillefer, Dentsply). The root canals were instrumented up to their apical constriction, under continuous manual irrigation with 1% NaOCl. During this process, a size-15 hand file was also used, to keep the apical foramen patent. After instrumentation to ProTaper F2, 5 ml of sterile physiological saline solution were administered to full working length to inactivate possible remnants of NaOCl. After drying the root canal with sterile paper points, a fine paper point (Orbis Dental, Münster, Germany) was inserted approximately 2 mm above the apical foramen to collect the apical tissue fluid, and the point was kept in that position for 30 s. Three consecutive paper points were collected from one canal and immediately after frozen at −80 °C until further processing.

2.2. Sample preparation

Initially, the paper point content was re-eluted in the tubes with 300 µl of sterile phosphate buffered saline (PBS), containing a protease inhibitor (complete mini EDTA free, Roche, Basel, Switzerland). The tubes were placed for 5 h on a platform shaker at (2000 rpm) at 4 °C, vortexed for 30 s, and thereafter centrifuged for 10 min at 5000 rpm. The resulting cell pellet was collected and processed for FISH analysis, as described further.

2.3. Analysis of apical samples by fluorescent in situ hybridization (FISH) and epifluorescence microscopy

The detection and counting of bacteria in the prepared samples was performed by FISH, followed by epifluorescence microscopy, in similar principles as previously described [8]. Briefly, 50 µl of 0.9% NaCl containing RNase inhibitor (Sigma Aldrich, Buchs, Switzerland) were added onto the pelleted sample, followed by shaking for 45 min, and vortexing for 1 min. Then, 10 µl of the suspensions were mixed with 5 µl of coating buffer (0.9% NaCl, 0.02% NaN_3, 2.5×10^{-4}% hexadecyltrimethylammonium bromide) on multi-well epoxy coated Adcell slides, with a well-diameter of 4 mm (Cel-Line, Erie Sientific Company, Portsmouth, NH, USA). The slides were air-dried and fixed by a 20 min-incubation in 4% paraformaldehyde at 4 °C, washed with nanopure H_2O and then processed for FISH analysis [8]. Every well was covered with 9 µl Denhardt's Solution (diluted 1:50 in PBS), including 1:500 RNase inhibitor, to reduce non-specific probe binding to the bacterial cell wall. The slides were then incubated for 30 min at 37 °C. Four specific oligonucleotide rRNA probes were used for *Synergistetes* cluster A and *Synergistetes* cluster B bacteria [6,8], a genus-specific probe for oral *Fusobacteria* [18], and a genus-specific probe for oral *Actinomyces* [19]. The cluster classification of oral *Synergistetes* bacteria into A and B was based on earlier studies [2,6,8]. Table 1 lists the oligonucleotide sequences and Cy3 or 6-FAM labeling of the used rRNA probes (Microsynth, Balgach, Switzerland), as well as their targeted taxa. The final probe concentrations used for FISH were 5 ng/µl for Cy3 conjugates and 20 ng/µl for FAM conjugates, in the presence of 40% formamide. For hybridization, 3–4 µl of probe solution was added to the wells, and incubated for 4 h, at 46 °C. Thereafter, they were washed for 30 min, air-dried and covered with 50 µl mounting fluid and a cover-slip. An Olympus BX60 fluorescence microscope (Olympus Optical AG, Volketswil, Switzerland) was used for the quantitative evaluation of the FISH stained samples. Fluorescence and direct light images of the detected bacteria were taken by an Olympus E510 camera. The quantitative evaluation of the stained bacterial taxa was done by counting the fluorescent bacterial cells in at least 10 viewing fields per well, at 100× magnification, as previously described [20]. The lowest detection limit of the assay was 50 bacterial counts per sample.

2.4. Statistical analysis

The Chi-square test was used to compare the frequencies of detection of the different bacterial taxa among the three clinical diagnosis groups. Statistical significance level was set at $P < 0.05$.

Table 1
16S rRNA-targeted probe sequences for FISH and target taxa.

Probe	Sequence (5′–3′)	Target taxa
SYN-A1409-FAM	ACACCCGGCTCGGGTGGT	*Synergistetes* cluster A
SYN-B1149-Cy3	TCGATGGCAGTCTCGCCG	*Synergistetes* cluster B
L-ACT476-2-FAM	ATCCAGCTACCGTCAACC	Genus *Actinomyces*
FUS664-Cy3	CTTGTAGTTCCGCYTACCTC	Genus *Fusobacterium*