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CENTRE
FOR
INTERNATIONAL HEALTH

A qualitative and quantitative study of five selected periodontal pathogens in combined periodontal-endodontic lesions

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Thesis submitted to the Faculty of Dentistry, University of Bergen in partial fulfilment of the requirements for the degree of Master of Philosophy in Dentistry

UNIVERSITY OF BERGEN – NORWAY
2005

ISBN 82-8006-025-1

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ACKNOWLEDGEMENTS

I would like to acknowledge:

My supervisor, Professor Nils Skaug, DDS, PhD, Head of the Laboratory of Oral Microbiology, Department of Oral Sciences, Faculty of Dentistry for his important help, advice and for providing all the necessary equipment and conditions.

Professor Vidar Bakken, Laboratory of Oral Microbiology, Department of Oral Sciences, Faculty of Dentistry for his help and advice.

Mrs. Brita Lofthus and Mrs. Øyunn Nielsen, for their prompt technical help.

Mr. Håvard Valvatne, for his advice concerning PCR techniques.

Professor Karl-Henning Kalland, Gades Institute for his advice concerning real-time PCR.

Mr. Geir Egil Eide, Centre for Clinical Research, Armauer Hansen Building and Mrs. Valborg Baste, Section for Epidemiology and Medical Statistics for statistical advice.

Professor emeritus Ronald Scheline, for prompt and professional linguistic comments on my thesis.

Kari and Øyvind Vågnes for their advice and help concerning many laboratory problems.

Collegues and staff at the Faculty of Dentistry.

Collegues and staff at the Centre for International Health.

My friends and colleagues: Daniela-Elena Costea, Mihaela Cimpan, Roxana Draghincescu, Delia Bancila, Irina Vamanu, Nezzar Al-Hebshi and Mohammed Al-Haroni.

This study was carried out while I was master student in the Oral Microbiology Department, Faculty of Dentistry, University of Bergen and Centre for International Health (2003-2005). The work received financial support from the University of Bergen and The Norwegian State Educational Loan Fund for Higher Education through the Quota Programme.

June 14, 2005
Bergen, Norway

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SUMMARY

It is well known that most periodontal pathogens are also endodontic pathogens. Little is known, however, about microflora of combined periodontal-endodontic lesions. Such coexisting pulpal and periodontal inflammation affecting the same tooth is relatively rare and can complicate the diagnosis and treatment planning of the involved tooth. **Objectives:** To investigate quantitatively and qualitatively five selected bacteria known as periodontal pathogens, in samples from combined periodontal-endodontic lesions and in a similar number of autologous samples from simple periodontal lesions. **Materials and methods:** Paired subgingival plaque and root canal samples from combined lesions and subgingival samples from 14 separate periodontal sites (reference sites) in 19 patients (12 women and 7 men; mean age 44±19.3 years) were collected using sterile paper points. The 52 plaque samples, 33 subgingival and 19 from root canals, were cultivated and processed for bacterial DNA extraction. *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythensis, Prevotella intermedia and Treponema denticola* were identified using conventional polymerase chain reaction (PCR). Heteroduplexes formation and analysis were used for identification of different *P. gingivalis* strains. Bacterial growth was assessed as the number of anaerobic colony forming units (CFU) per sample. A Taqman-based® real-time PCR protocol was used for quantification of total bacteria, *P. gingivalis* and *A. actinomycetemcomitans*. The Wilcoxon signed rank test and McNemar tests were used to evauate the statistical significance of differences between the paired samples. Accordance between the different microbiological methods (conventional PCR versus real-time and PCR and culture versus real-time PCR) was tested using Kappa and Spearman‘s correlation tests. Association between the studied pathogens was evaluated using the chi-square test. P-values < 0.05 were considered statistically significant. **Results:** Both cultivation and real-time PCR showed significantly (p=0.048 and p=0.0154) higher numbers of bacteria in subgingival plaque samples from combined lesions than in those from simple periodontal lesions. After real-time quantification, the proportions of *P. gingivalis* and *A. actinomycetemcomitans* in total number of bacteria were calculated. No significant difference was demonstrated between combined and simple lesions. Conventional PCR demonstrated 57.9%, 21.1% and 42.86% positive samples for *A. actinomycetemcomitans* 78.9%, 42.1% and 78.6% for *P. gingivalis*, 42.11%, 21.1% and 35.7% for *P. intermedia*, 68.4%, 21.1% and 64.3% for *T. forsythensis,* and 47.4%, 26.3% and 35.7% positive samples for *T. denticola* in the three types of samples, respectively. Periodontal samples from combined periodontal-endodontic lesions demonstrated significant associations between *P. gingivalis* and *T. denticola* (p=0.033) and between *P. gingivalis* and *T. forsythensis* (p=0.035). From all the samples that tested positive for *P. gingivalis,* heteroduplexes analysis revealed that 18 of these contained one strain, 14 showed two strains and only two had three different strains. **Conclusions:** The significantly higher bacterial levels in subgingival plaque samples from combined periodontal lesions indicate bacterial migration between the root canal and the periodontal pocket. The positive correlation between the bacterial levels of subgingival and endodontic samples from combined periodontal-endodontic lesions also indicates a bacterial communication between the two compartments. The presence of the same strain(s) in periodontal and endodontic samples from combined lesions support the idea that such lesions may represent a single pathological entity.

INTRODUCTION

The periodontal-endodontic controversy

Over the past century the dental literature has consistently reflected a controversy regarding the effect of periodontal disease on the dental pulp and more recently the effect of pulpal necrosis on the initiation and progression of marginal bone loss. It has been reported that many dental practitioners referred incorrectly to combined periodontal-endodontic (perio-endo) lesions if bone resorption is evident radiographically in the furcation or crestal area or gave the disease process an incorrect designation simply because bone resorption extended to the apex of the affected tooth (1).

The International Workshop for the Classification of Periodontal Diseases and Conditions adopted in 1999 the latest classification for periodontal diseases and conditions and a category of periodontitis associated with endodontic lesions and a subcategory of combined perio-endo lesions was added to the classification (2).

Both endodontic and periodontal diseases are caused by mixed anaerobic infections (see below). Although the topographical relationship between the dental pulp and the periodontium is well documented, the pathways for the spread of bacteria between pulpal and periodontal tissues have been discussed and are controversial (3, 4). The interrelationship between pulpal and periodontal disease occurs via the intimate anatomical and vascular connections between the pulp and the periodontium. This interrelationship has been traditionally demonstrated using radiographic, histologic and clinical criteria. As the tooth develops and the root is formed, the anatomical-physiological pathways for communication are created. Accidental (non-physiological) pathways may also occur. Bacteria and inflammatory products can pass through these perio-endo pathways.

Endodontic-periodontal communications

Physiological pathways

The apical foramen is the direct route of communication between the pulp and the periodontium. The apex is also a portal of entry to the pulp from deep periodontal pockets. Pulp inflammation or pulp necrosis extends into the periapical tissues causing a local inflammatory response accompanied by bone and sometimes by root resorption (5).

Dentinal tubules contain cytoplasmatic extensions or odontoblastic processes that extend from the odontoblasts at the pulp and dentin border to the dentin and enamel junction or dentin and cementum junction. The number of dentinal tubules per square millimetre varies from 8,000 to 57,000. At the periphery of the root at the cemento-enamel junction, the number has been estimated to be approximately 15,000 per mm^2 (6). Exposure of dentinal tubules may occur due to developmental defects, disease or periodontal treatment procedures. Scanning electron microscopic studies have demonstrated that dentin exposure at the cemento-enamel junction occurs in 18% of teeth in general and in 25% of anterior teeth in particular (7, 8). It was demonstrated that bacteria are present in root dentinal tubules of teeth with apical periodontitis (9).

Lateral and accessory canals appear mainly in the apical area and in the furcation of molars and connect also the pulp with the periodontal ligament. These have been suggested as a direct pathway between the pulp and periodontium and they typically contain connective tissue and blood and lymph vessels that connect the circulatory systems of the two tissues. It is estimated that 30-40% of all teeth have lateral or accessory canals. The presence of open accessory canals is a potential pathway for the spread of bacterial and toxic products from the pulp, resulting in an inflammatory process in the periodontal ligament (10). The dental pulp is usually not directly affected by periodontal disease until the gingival recession opens an accessory canal to the oral environment (5).

Palatogingival grooves are developmental anomalies of the maxillary incisor teeth, with lateral incisors more often affected than central ones. These grooves usually begin in the central fossa, cross the cingulum and extent apically at varying distances (5).

Non-physiological (accidental) pathways

Perforation of the root creates an artificial communication between the root canal system and the periodontal ligament. This may occur as a result of overinstrumentation during endodontic procedures, internal or external resorption or caries invading through the floor of the pulp chamber. The closer the perforation is to the gingival sulcus/ periodontal pocket, particularly into the coronal third of the root or the furcation region, the greater is the

likelihood of an apical migration of the gingival epithelium in initiating a periodontal lesion (11).

Vertical root fracture occurs occasionally and can be visible radiographically as a "halo" effect around the affected tooth. The fracture site provides a portal of entry for bacteria and their toxic products, fungi and viruses, as well as for nonliving agents such as foreign bodies, cholesterol, Russell bodies, Rushton hyaline bodies and Charcot-Leyden crystals from the root canal system to the surrounding periodontal ligament (5, 11). Figure 1 illustrates the bacterial pathways between the pulp and periodontium.

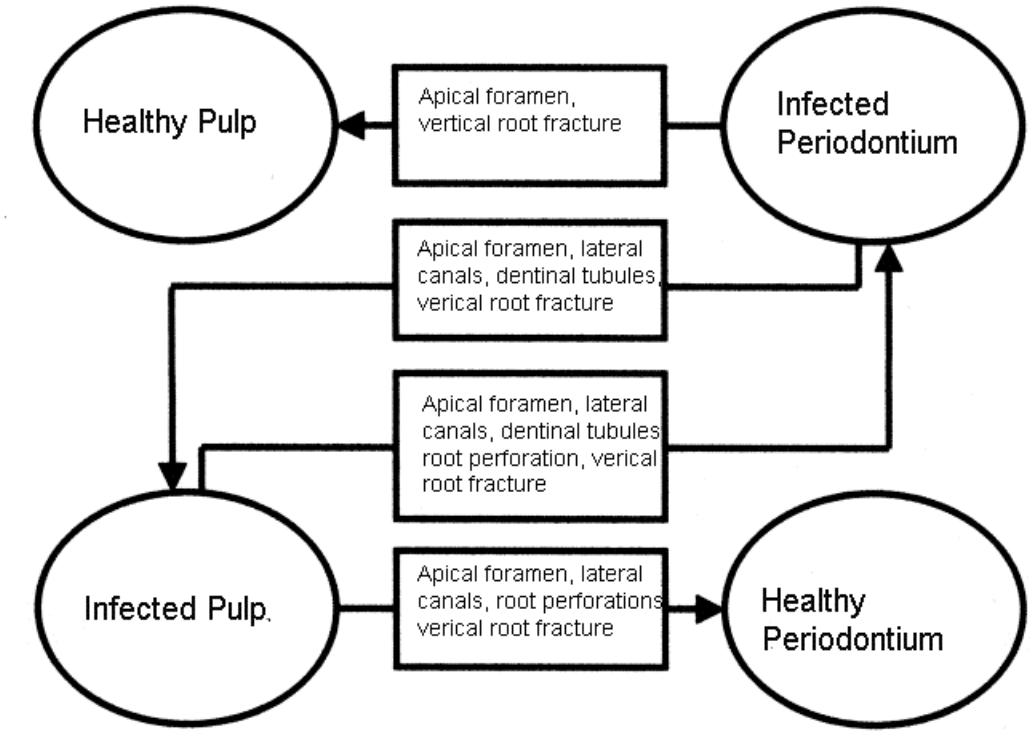


Fig 1. The possible directions for infection spreading between the dental pulp and periodontium (4).

Classification of periodontal-endodontic lesions

The first classification of perio-endo lesions based on etiology, diagnosis and prognosis of the involved tooth, was proposed by Simon et al. (1972) (12) as follows:

1. Primary endodontic lesions
2. Primary periodontal lesions

3. Primary endodontic lesions with secondary periodontal involvement
4. Primary periodontal lesions with secondary endodontic involvement
5. True combined lesions

In this thesis, group 3, 4 and 5 above are referred to as combined perio-endo lesions. Other authors added one category called "Independent periodontal and endodontic lesions" (6) or "Concomitant pulpal and periodontal lesions" (10, 13). For a primary endodontic with secondary periodontal involvement Shilder and Grossman (1988) (14) used the term "Lesion of endodontic origin". Recently, von Arx and Cochran (2001) proposed a clinical treatment classification of perio-endo-furcation lesions based on the role of membrane application in endodontic surgery (15).

In contrast to combined perio-endo lesions, concomitant pulpal and periodontal lesions reflect the presence of two separate and distinct disease states with different causative factors and with no clinical evidence that one disease state has influenced the other.

Etiology

Bacteria, fungi and viruses represent live pathogens encountered in a diseased pulp and periapical tissues. Both periodontal and endodontic infections are polymicrobial and biofilm-related comprising anaerobic and facultative anaerobic bacteria.

Pathogenesis

Endodontic infections

Most pathoses of the dental pulp and periradicular tissues are either directly or indirectly related to microorganisms. Bacteria may take several routes to invade and infect the pulp. In addition to the pathways presented above, anachoresis (transport of microorganism by blood to the area where they establish an infection) can contribute to pulpal and periradicular infections (16). The condition of the pulp is an important factor in susceptibility to microbial invasion. A vital pulp is very resistant to microbial invasion. Penetration of the surface of a healthy pulp by oral bacteria is relatively slow or may be blocked entirely. In contrast, a necrotic pulp is rapidly invaded and colonised by bacteria (5).

Of about 500 species of bacteria until recently recognised as normal oral flora, only a relatively small group is commonly isolated from infected dental pulp cavities. Strictly

anaerobic bacteria predominate (more than 90%), with some facultative anaerobes and, rarely aerobes. This suggests a selective process favouring the growth of anaerobes. The relative proportion of strict anaerobic bacteria to facultative aerobic bacteria increases with time, as does the total number of bacteria (16, 17). According to the most recent report, it is presumed that over 700 bacterial species (phylotypes included) inhabit the oral cavity and more than half of these cannot be cultivated (18-20). Specific combinations of bacteria are found in the root canal and they can contribute to ecological shifts of the flora by different mechanisms of interaction (21).

Periodontal infections

Infectious periodontal diseases are caused by microorganisms colonizing the tooth surface at or below the gingival margin. Bacteria may attach to the tooth itself, to the epithelial cells of the gingiva or periodontal pocket, to underlying exposed connective tissues and to other bacteria which are attached to these surfaces. Microbial complexes colonizing the subgingival area as biofilm can provide a variety of relationships with the host, ranging from preventing to causing the disease. Periodontal disease is dependent on the simultaneous occurrence of a number of factors for initiation and progression such as virulent periodontal pathogens, local environment and host susceptibility. It is now known that about 15 host genes are involved in periodontal disease in humans (22).

There is no single cause to periodontal infections and not single treatment can control the disease.

It has been shown that subgingival plaque contains about 350 cultivable bacterial species and around 100 of these can be found in samples from a particular individual (23).

Different studies that used criteria such as association between pathogens and disease, elimination of species and parallel remission of disease, host response, virulence factors, animal models, checkerboard DNA-DNA hybridisation and PCR, made possible to associate different bacteria with different periodontal pathoses. *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Tanerella forsythensis, Prevotella intermedia, Fusobacterium nucleatum, Campylobacter rectus, Eikenella corrodens, Peptosreptococcus micros, Eubacterium* species are the best documented periodontal pathogens (24). Cluster analysis of subgingival plaque has demonstrated that certain species

frequently occur together in complexes (25). “Red” and “orange” complex species appear more frequently subgingival, while “green” and “purple” complex species are more common supragingival (26).

Lately different types of viruses were discovered in periodontal infections and it was suggested that the coexistence of periodontal HCMV, EBV and possibly other viruses, periodontopathic bacteria, and local host immune responses should be viewed as a potential cause for periodontal destruction (27).

Combined periodontal-endodontic infections

The literature contains no description of the periodontal-endodontic lesion microbiology. Due to great similarity between the microbiota of periodontal and endodontic lesions, the periodontal-endodontic lesion might show no unique microbiological profile (28). Rupf et al. (1999) studied the profiles of periodontal pathogens in pulpal and periodontal disease associated with the same tooth and they discovered comparable profiles of periodontal pathogens in pulpo-periapical disease except the “progressive adult periodontitis” group (29). Kobayashi et al. (1990) paper suggested that the periodontal pocket may be a possible source of root canal infections (30).

Clinical considerations

Primary endodontic lesions (Figure 2a)

An acute exacerbation of a chronic apical lesion in a tooth with a necrotic pulp may drain coronally through the periodontal ligament into the gingival sulcus and mimic clinically the presence of a periodontal abscess. In reality it is a sinus tract from pulpal origin that opens through the ligament area. A similar situation occurs where drainage from the apex of a molar tooth extends coronally into the furcation area or in the presence of lateral canals extending from a necrotic pulp into the furcation area. Usually the lesion will heal following root canal treatment.

Primary periodontal lesions (Figure 2b)

These lesions are caused primarily by periodontal pathogens. In this process, chronic periodontitis progresses apically along the root surface. In most cases, pulp-vitality tests will indicate a clinically normal pulpal reaction. Periodontal disease has a progressive nature. It

begins in the sulcus and migrates towards the apex as deposits of plaque and calculus produce inflammation, causing loss of surrounding alveolar bone and supporting periodontal tissue. The bony lesion is usually more widespread and generalised than are lesions of endodontic origin. Because this is a purely periodontal problem, the prognosis depends exclusively on the outcome of periodontal therapy.

Primary endodontic lesions with secondary periodontal involvement (Figure 2c)

Such lesions appear if the primary endodontic disease remains untreated. It will continue, leading to destruction of the periapical alveolar bone and progressing into the interradicular area, causing breakdown of surrounding hard and soft tissues. As drainage persists through the gingival sulcus the accumulation of plaque and calculus in the purulent pocket results in periodontal disease and further apical migration of the attachment. When this occurs not only does the diagnosis become more difficult, but the prognosis and treatment also are altered. Resolution of the primary endodontic and secondary periodontal lesion relies on treatment of both conditions. If endodontic therapy is adequate, the prognosis will depend on the severity of periodontal involvement and efficacy of periodontal therapy.

Primary endodontic lesions with secondary periodontal involvement may also be the results of root perforation during root canal treatment, root fracture or misplacement of pins or posts during coronal restorations (5).

Primary periodontal lesions with secondary endodontic involvement (Figure 2d)

Periodontal disease may have an effect on the pulp through dentinal tubules, lateral canals or retrogradly from apex. Also, lateral canals and dentinal tubules may be opened to the oral environment by scaling and root planning or surgical flap procedures. However, teeth that become necrotic as a sequeal of periodontal disease are very rare (31). If the tooth does not respond to periodontal treatment, a necrotic pulp may be the cause. Once the pulp becomes inflamed, it can in turn affect the primary periodontal lesion. Radiographically, these lesions may be indistinguishable from the primary endodontic lesions with secondary periodontal involvement. The prognosis depends on continuing periodontal treatment subsequent to endodontic therapy.

True combined lesions (Figure 2e)

Pulpal and periodontal diseases may occur independently and concomitantly in and around the same tooth. Once the endodontic and periodontal lesions coalesce, they may be clinically indistinguishable.

The typical combined perio-endo lesion that can be identified presents radiographic evidence of bone loss, which appears to extend some distance down the lateral root surface from the crestal bone. Probing reveals the typical conical periodontal type of probing with the exception that at the base of the periodontal lesion, the probe will abruptly drop further down the lateral root surface and may even extend to the apex of the tooth. The lesion can be characterized as a typical sinus tract type of defect at the base of a periodontal lesion (6).

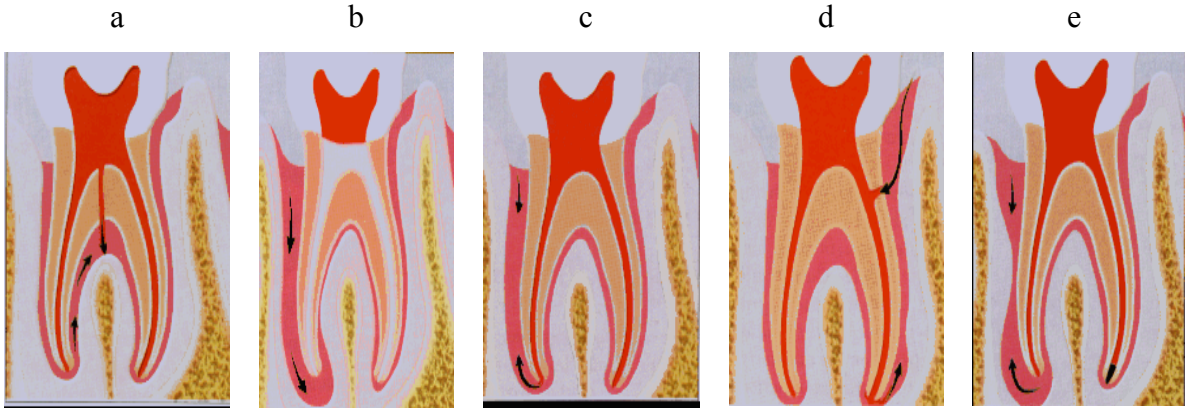


Fig 2. Illustrations modified from John et al. (2004) (32) of combined periodontal-endodontic lesions according to the Simon et al. (1972) classification (12).

Diagnosis

A tooth with a combined perio-endo lesion must fulfil the following criteria:

- The tooth involved must be pulpless (avital pulp or incomplete root filling)
- There must be destruction of the periodontal attachment that can be diagnosed by probing from the gingival sulcus to either the apex of the tooth or to the area of an involved lateral or accessory root canal.
- Both root canal treatment and periodontal therapy are required to resolve the entire lesion (6, 10).

The diagnosis of periodontal lesions associated with pulpal disease may be relatively simple if a patient has been monitored over a period of time and records are available. The diagnosis

is more difficult when a complete history is unavailable. A growing periapical lesion with secondary formation of a deep periodontal pocket may be similar in clinical and radiographic appearance to a longstanding periodontal lesion that has progressed to the apex. The radiographic image of bone resorption, including the apical and furcal or marginal regions, may confuse rather than aid in making a diagnosis. However, if radiographs taken during the progression of bone resorption reveal it to be extending from the apex to the bone crest, the apical region can be positively identified as the origin of the infection. In general, it is easier to determine the origin of the lesion when a vitality pulp test is positive, because this will rule out an endodontic aetiology. However, pulp tests may not be always reliable. This consideration is particularly relevant when challenges to pulpal status arise from periodontal diseases. Partial necrosis of a pulp can appear especially in a multirooted tooth. This may allow positive responses to pulp testing suggesting vitality, despite the existence of a combined lesion.

A non-vital or endodontically-treated tooth associated with a periodontal lesion presents a greater diagnostic problem. In this situation, pulpal necrosis is frequently associated with inflammatory involvement of the periodontal tissue. The location of these pulpal lesions is most often at the apex of the tooth, but they may also occur at any site where lateral and furcal canals exit into the periodontium (11).

Clinical diagnosis

Clinical tests are imperative for obtaining correct diagnosis and differentiating between endodontic and periodontal disease. Different signs and symptoms can be assessed by visual examination, palpation, and percussion. Mobility testing brings data about the integrity of the attachment apparatus or to the extent of inflammation in the periodontal ligament. Teeth with extreme mobility generally have little periodontal support, indicating that the primary cause may be periodontal disease.

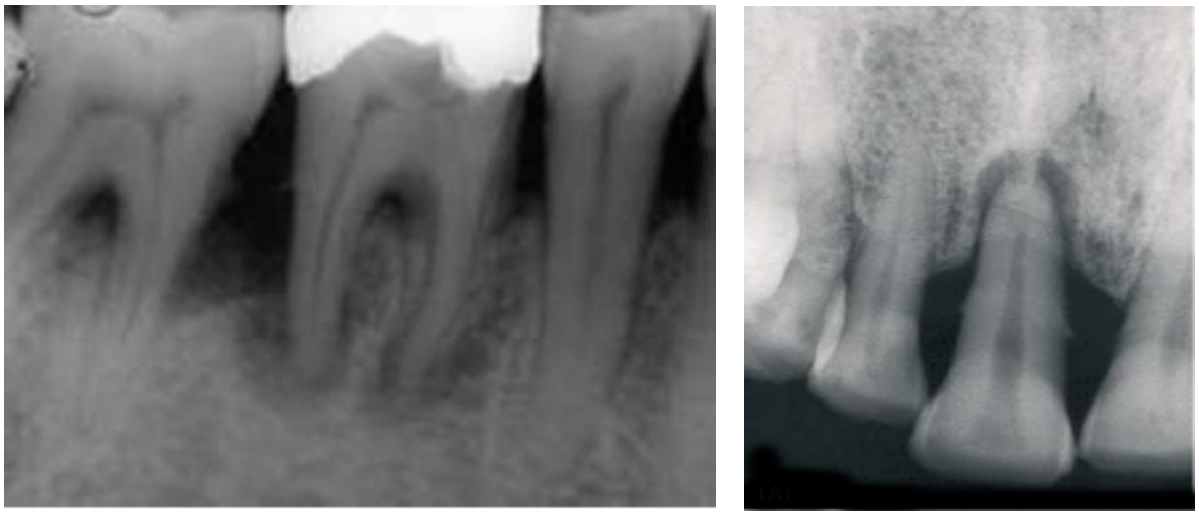
Radiographs

Radiographs are essential for detection of anatomic structures and pathological conditions. They aid in detection of carious lesions, extensive or defective restorations, pulp caps, pulpotomies, previous root canal treatment and possible mishaps, stages of root formation,

canal obliteration, root resorption, root fractures, periradicular radiolucencies, thickened periodontal ligament and alveolar bone loss.

The integrity of the dental pulp cannot be determined by radiographic images alone. Often, the initial phases of periradicular bone resorption from endodontic origin are confined only to cancellous bone. Therefore it cannot be detected unless the cortical bone is also affected. On the other hand, periodontal disease causing alveolar bone loss can be effectively detected by radiographs (see Figure 3).

For purpose of differential diagnosis, periapical and bitewing radiographs that are taken from several incidences are used (5).



a **b**

Fig 3. a) Combined perio-endo lesion in a mandibular first molar; joined endodontic and periodontal lesions. b) Combined perio-endo lesion in central incisor with bone loss in two-third of the root and apical bone resoption. (5)

Pulp vitality tests

Determination of pulp vitality is essential for a correct differential diagnosis and for selection of primary measures for treatment of inflammatory lesions in the marginal and apical periodontium. An abnormal response may indicate degenerative changes in the pulp. When these tests are correctly performed and adequately interpreted, they are reliable in differentiating between pulpal disease and periodontal disease. The most commonly used pulp vitality tests are: cold test, electric test, blood flow test and cavity test.

Pocket probing

Periodontal probing is an important test for differential diagnosis and for prognostic aid. It offers data about location and extent of pockets, probing depth and furcation invasions. Also the presence of subgingival calculus deposits and the degree and location of inflammation are important to note in assessing the primary source of the disease.

Fistula tracking

Endodontic or periodontal disease may sometimes develop a fistulous sinus track. Identifying the origin of inflammation by tracking the fistula will help the clinician to differentiate the source.

Microbiological examination

In special cases microbiological analysis can provide useful information for the diagnosis and treatment. Several different types of microbiological tests are available. Usually the tests are used for patients who have not responded favourably to conventional therapy or for monitoring patients on maintenance therapy for recurrence of disease.

The information generated by microbiological analysis of plaque collected from patients is highly dependent on the sampling technique. Usually plaque samples are collected from root canals using sterile paper points. For subgingival plaque there are two primary sampling methods: using curettes and adsorption on endodontic paper points. Information gathered from curette and paper point samples differs (25, 33, 34).

Microbiological tests for plaque samples

A variety of techniques for analysing plaque samples have been developed. These include microscopy, bacterial culture, enzymatic assays, immunoassays, nucleic acid probes and polymerase chain reaction (PCR) assays. Microscopic identification uses brightfield, darkfield, phase contrast and scanning electron microscopy for assessment of plaque samples. Such visual techniques used for gram-stained smears or native specimens can determine the relative proportions of visible organisms. Since it is not possible to identify individual species in this way, the main usefulness comes in observing shift in the appearance of the flora (35, 36).

Microbiological culture methods were considered the gold standard for the other microbiological identification methods (28, 37). Many perio- and endopathogenic micro-organisms can be identified by the use of selective and non-selective media, often in combination with biochemical tests for speciation. However, these methods also have significant limitations like inability to detect low levels of micro-organisms, high cost, labour intensiveness, prolonged time before results and difficulty in growing several bacterial species. Also, molecular methods have demonstrated about 200 oral phylotypes that have so far not been cultivated (20).

Immunoassays identify bacteria using monoclonal or polyclonal antibodies against species-specific antigens.

Nucleic acid probes (DNA or RNA) consist of nucleic acid sequences that are labelled with radioactive or enzymatic-colorimetric markers that bind to complementary nucleic acid sequences on corresponding micro-organisms.

PCR is a molecular technique for high-yield replication of DNA. It allows synthesis of vast number of copies from minute samples of DNA. For the detection of oral pathogens, PCR is an establish method (38-43). It permits also detection of different multiple strains (44-46). Real-time PCR is a good alternative to conventional PCR for the study of bacterial load because of its low inter-assay and intra-assay variability and improved sensitivity compared to a microbial culture or conventional single-round and nested PCR (47, 48). Real-time PCR has been reported to be at least as sensitive as Southern blot that is still considered by some as the gold standard for probe-based hybridisation assays (49).

Quantitative real-time PCR is an important tool for nucleic acids concentration. The product accumulation is monitored during the PCR process in real time by fluorescence technique. This technique combines high sensitivity with a high dynamic range for quantification without the requirement of a post-PCR analysis. The fluorescence signal curves from the individual amplification reactions are used to determine Ct values (crossing point of the signal curves with an arbitrary threshold). If the threshold intersects the amplification curves in their exponential phase, the Ct values are proportional to the logarithm of the initial copy number. Using the information of Ct values relative quantification is possible using the ΔΔCt method (50).

Treatment and prognosis

Treatment decision-making and prognosis depend primarily on the diagnosis of the specific endodontic and or periodontal disease. The prognosis of multirooted teeth with combined perio-endo lesions depends largely on the extent of the destruction caused by the periodontal disease component. A necrotic pulp or a failing endodontic treatment, plaque, calculus, and periodontitis will be present in varying degrees. In general, assuming that the endodontic therapy is adequate, what is of endodontic origin will heal. Thus the prognosis of combined disease depends on the efficacy of periodontal therapy. Some authors think that by identifying the physical contour of the lesions in the attachment by careful probing and accurately interpreting the pulp test responses it can be determined which defects can be resolved by root canal treatment and which cannot.

RATIONALE OF THE STUDY

The subject of bacterial migration between the root canal and periodontium is still under discussion despite many studies and there is little information about the microbiology of combined lesions (3, 4). However, the similarities between the endodontic and periodontal microflora suggest that cross-infection between the root canal and the periodontal pocket can occur (4). The simultaneous existence in the same tooth of pulpal problems and inflammatory periodontal disease can complicate diagnosis and treatment planned for the affected tooth and in my clinical experience treating such cases proved to be a challenge.
A recent review paper (Zehnder et al. 2002) (4) concludes that the need for new data cannot be overemphasized.

AIMS

The objectives of the study were:

- To quantify some selected pathogens in samples from combined lesion.
- To identify individual bacterial species in the samples and to see whether they are of the same or different clonality.
-To compare the subgingival flora of combined lesions and single periodontal lesions with respect to quality/quantity of pathogens.

MATERIALS

Plaque samples and patients

Paired subgingival and root canal samples and samples from 14 separate periodontal sites (total 52 plaque samples; 33 subgingival and 19 root canal samples) were collected from 19 patients (12 women and 7 men; mean age 44±19.3) treated at the Periodontal and Emmergency Departments, the University Hospital of Stomatology ”Prof. Dr. Dan Theodorescu”, Bucharest, Romania. The patients participated on the basis of oral information about the sample collection and informed consent.

Tooth selection

Inclusion criteria

The criteria for inclusion of the teeth used for root canal and subgingival plaque sampling were:

-need for booth periodontal and endodontic treatment

-closed pulp cavity before sample collection

-presence of pulp necrosis

-presence of chronic periodontitis (pocket depths ≥ 4mm)

-no previous endodontic and/or periodontal treatment

Pulp necrosis was diagnosed by vitality tests. Periodontal disease was assessed by pocket probing and X-ray.

Exclusion criteria

Patients who received antibiotic treatment within the preceding three months and teeth that could not be suitably isolated were excluded from the study.

Materials used for collection of samples

- Sterile paper points, ISO number 20 and 50 (Dentsply Maillefer) (Figure 4)
- Sterile 2ml capped microtubes with an O-ring (PP Sarstedt Germany) that were DNA and RNA free and contained 1ml sterile 5% dimethyl sulfoxide in thrypticase-soy broth TSB-DMSO (Difco, Detroit, MI, USA) and 0.5g sterile glass beads. (Figure 4)

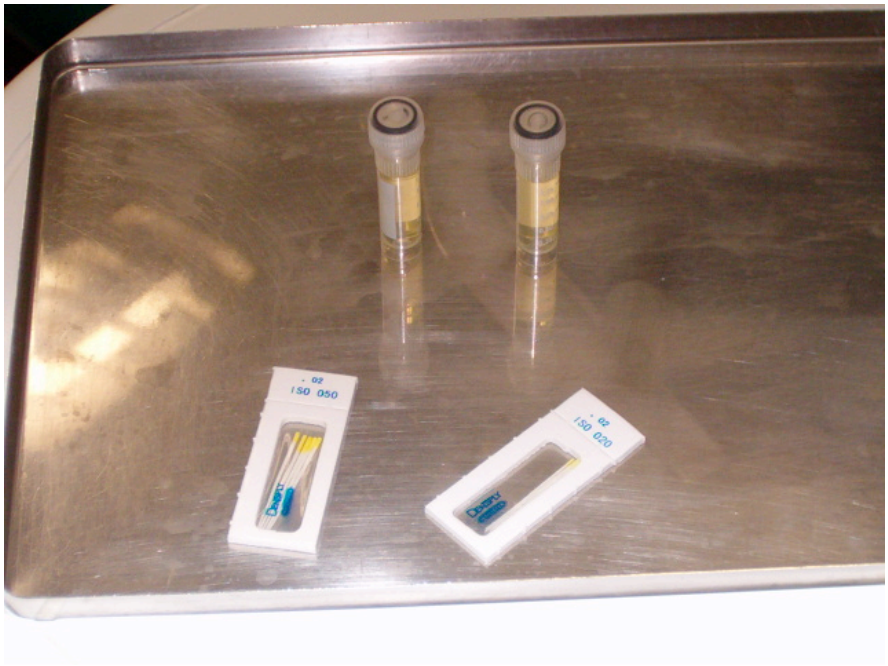


Fig 4. Two packages with sterile paper points and two tubes used for sample collection.

Bacterial strains

The following bacterial strains available in the Laboratory of Oral Microbiology, the Faculty of Dentistry, University of Bergen were used for cultivation and as standards for the PCR procedures:

-*Porphyromonas gingivalis* ATCC* 33277, ATCC 53978 -W50

-*Actinobacillus actinomycetemcomitans* ATCC 33384, ATCC 43717 and ATCC 43718

-*Prevotella intermedia* ATCC 25611- VPI**4197, VPI 4202 and VPI 4196

-*Tannerella forsythensis* ATCC 43037- FDC*** 338, FDC 42 and FDC 2008

*ATCC = American Type Culture Collection, Rockville Maryland, USA

**VPI = Virginia Polytechnic Institute and State University, Blacksburg Virginia, USA

***FDC = Forsythe Dental Center, Boston, USA

Cultivation media used:

- Anaerobic blood agar plates (Fastidious Anaerobe Agar, Lab M, UK) (see Appendix I).

-Trypticase soy-bacitracin-vancomycin (TSBV) plates (see Appendix I).

- Chemostat fluid medium with vitamin C (see Appendix I).

DNA extraction kit

The QIAamp DNA Mini Kit (Qiagen- Germany) was used.

The following primers and master mix were used for conventional PCR:

Primers

***Actinobacillus actinomycetemcomitans* (*A.a.*)**

- Universal forward (785) 5'-3': GGA-TTA-GAT-ACC-CTG-GTA-GTC
- Universal reverse (422) 5'-3': GGA-GTA-TTT-AGC-CTT
- *A.a.* forward (AS2) 5'-3': GGT-AAC-CAA-CCA-GCG-ATG-GG
- Universal reverse (241) 5'-3': TTC-GCT-CGC-CGC-TAC-T

***Porphyromonas gingivalis* (*P.g.*)**

- Universal forward (785) 5'-3': GGA-TTA-GAT-ACC-CTG-GTA-GTC
- Universal reverse (422) 5'-3': GGA-GTA-TTT-AGC-CTT
- *P.g.* forward (PG3R) 5'-3': CGA-TAT-ACC-GTC-AAG-CTT-CCA-CAG
- Universal reverse (L189) 5'-3': GGT-AAT-GAG-ATG-TTT-CAG-TTC
- *P.g.* forward (PG7R) 5'-3': CCG-CAA-GGG-GCG-CAC-TAG-GGT-AAT
- Universal reverse (EricM) 5'-3': GCC-TAG-GCA-TCC-ACC-G

***Treponema denticola* (*T.d.*)**

- *T.d.* forward 5'-3': TAA-TAC-CGA-ATG-TGC-TCA-TTT-ACA
- *T.d.* reverse 5'-3': TCA-AAG-AAG-CAT-TCC-CTC-TTC-TTC-TTA

***Prevotela intermedia* and *Tannerella forsythensis* (*P.i.* and *T.f.*)**

- Forward 5'-3': AGA-GTT-TGA-TCC-TGG-CTC-AG
- *P.i.* reverse 5'-3': GTT-GCG-TGG-ACT-CAA-GTC-CGC-C
- *T.f.* reverse 5'-3': GTA-GAG-CTT-ACA-CTA-TAT-CGC-AAA-CTC-CTA

Master mix

The HotStarTaq Master Mix (Qiagen)

All conventional PCR reactions were run using the **ABI 96-Well GeneAmp® PCR System 9700 (Applied Biosystem International).**

Real-time PCR

Primers and probes

Universal

- Forward 5'-3': CGC-TAG-TAA-TCG-TGG-ATC-AGA-ATG
- Reverse 5'-3': TGT-GAC-GGG-CGG-TGT-GTA
- Probe 5'-3': YY-CAC-GGT-GAA-TAC-GTT-CCC-GGG-C-DarkQuencher

Amplicon size: 69bp

Actinobacillus actinomycetemcomitans

- Forward 5'-3': ACG-CAG-ACG-ATT-GAC-TGA-ATT-TAA
- Reverse 5'-3': GAT-CTT-CAC-AGC-TAT-ATG-GCA-GCT-A
- Probe 5'-3': 6FAM-TCA-CCC-TTC-TAC-CGT-TGC-CAT-GGG-TAMRA

Amplicon size: 77bp

Porphyromonas gingivalis

- Forward 5'-3': CCT-ACG-TGT-ACG-GAC-AGA-GCT-ATA
- Reverse 5'-3': AGG-ATC-GCT-CAG-CGT-AGC-ATT
- Probe 5'-3': 6FAM-TCG-CCC-GGG-AAG-AAC-TTG-TCT-TCA-TAMRA

Amplicon size: 71bp

Master mix

TaqMan® Universal PCR Master Mix, No AmpErase® UNG **ABI(Applied Biosystem International)**

The real-time PCR was performed using 7500 Real-Time PCR System **ABI (Applied Biosystem International).**

Electrophoresis was performed using:

- Agarose 1-2% (Seakem LE Agarose, FMC Bioproducts, Rockland, Maine. USA) in 1x TAE buffer tris-acetate-EDTA pH 8.5.
- Acrylamide CRITERION TBE 5% gels in 1x TBE buffer tris-boric acid-EDTA.

- DNA marker 50µg pGEM® DNA Markers, Promega USA was used for all electrophoresis procedures and has the following band pattern (Figure 5).

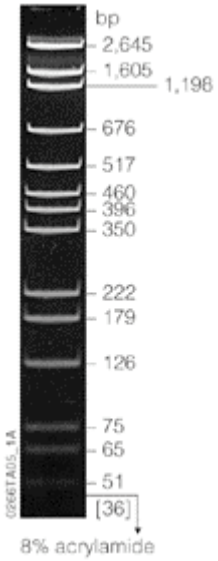


Fig 5. Electrophoresis lambda DNA marker.

METHODS

Collection of samples

Periodontal samples

Saliva was removed from teeth and the gingival margin with a cotton roll or gauze pad to reduce contaminating bacteria. The sample sites were then isolated with cotton rolls and air-dried, the supragingival plaque was removed and two #50 paper points were inserted as far as possible into the pocket. In the case of simple periodontal lesions the deepest pockets were chosen. After 1 minute they were removed and the two paper points from each subject were pooled and stored frozen at -70˚C (within one hour from the collection) in a sterile 2-ml microtube filled with 1 ml TSB-DMSO until further analysis.

Endodontic samples

The teeth to be sampled were first isolated using cotton rolls and then disinfected by swabbing with 2.5% sodium hypochlorite for 1 minute. The disinfectant was air dried before access was gained to the pulp chamber and root canal(s). Caries and/or existing restorations were removed. Then the cavity was disinfected with a sterile cotton pellet slightly wetted with 2.5% sodium hypochlorite (51). Finally the pulp chamber was accessed with a new sterile bur without water spray. If the canal was wet, it was filed superficially with a #15 file to release debris and bacteria. If it was dry, a small amount of sterile saline was introduced into the canal. The debris-laden fluid in the canal was then soaked up using sterile paper points. Two sterile #20 paper points were used to obtain the sample from each canal. In multicanaled teeth, one paper point sample was obtained from each accessible canal. If not accessible, the canal in the root with the largest periapical lesion and the largest canal were chosen. The paper points were left in position for 1 minute before they were transferred (within 1 hour) to a sterile microtube filled with 1ml TSB-DMSO and frozen at -70˚C.

Samples processing for cultivation

The samples in TSB-DMSO were thawed at 37˚C for 10 minutes and vortexed for 30 seconds and diluted 10-fold in 100µl chemostat fluid medium with vitamin C 1% (see Appendix I). Ten µL from each dilution was plated on anaerobic blood agar plates (Fastidious Anaerobe Agar, Lab M, UK) and Trypticase soy-bacitracin-vancomycin (TSBV) plates (see Appendix I). The anaerobic plates were incubated at 37ºC anaerobically (80% N_2, 10% CO_2 and 10% $H_{2)}$ using the Anoxomat System^{TM} (MART Microbiology BV, The Netherlands). After 7-10 days of incubation total anaerobic counts were assessed. The TSBV plates were incubated in air with 5% CO_2 at 37ºC (Anoxomat System^{TM}) for 5 days.

All the samples were cultivated in triplicate.

Samples processing for PCR

Two different methods of sample preparation for PCR were tested with samples from pure cultures of reference strains (see above) and four subgingival plaque samples from healthy volunteers.

Samples in TSB-DMSO at -70ºC were thawed at 37ºC for 10 minutes and vortexed for 30 seconds and treated in two different ways in a pilot study.

1. **Boiling**

The microbial suspensions were washed three times with 100µl of bidistilled water and centrifuged for 3 minutes at 3000xg. The pellets were then resuspended in 100µl of bidistilled water, boiled for 10 minutes and chilled on ice. After centrifugation at 4ºC for 10 seconds at 9000xg to remove the cell debris, the supernatant was collected for testing (52-55).

2. **QIamp DNA Mini Kit protocol (**Qiagen GmbH, Hilden, Germany)

Samples were centrifuged for 10 minutes at 5000xg and the bacterial pellet was suspended in 180µl of enzyme solution (20mg/ml lysozyme) and incubated for 30 minutes at 37ºC. Twenty µL of proteinase K stock solution (see Appendix II) and 200µl buffer AL were added. The samples were mixed by vortexing and incubated at 56ºC for 30 minutes and at 95ºC for 15 minutes.

Afterwards isolation of DNA was performed as recommended by Qiagen in tissue protocol for QIamp Mini Kit from step 4 (see Appendix II) (56).
Qiagen protocol was used for sample processing for PCR (40, 43).
The DNA yield, concentration and purity were determined using a spectrophotometer GeneQuant (Amersham Pharmacia Biotech Ltd. Cambridge, UK).
The results of the pilot study showed that the best DNA purity and concentration were obtained using the QIamp Mini Kit Qiagen and this method was used for all clinical samples and samples from bacterial strains.
The DNA yielded ranged from 22.4 to 79µg/ml for stock bacterial strains used as positive controls and from 3.9 to 48.7µg/ml for the clinical samples.
The extraction yielded 200µl that were stored in sterile, DNA-, RNA- free tubes and frozen at -20ºC until used.

Detection of *A. actinomycetemcomitans*

The samples were examined for the presence of *A.a.* using a nested PCR procedure.
Sequence of 16S ribosomal RNA was obtained from GeneBank (ascension number X90833 and U07777). Primers were checked with web Basic Local Alignment Search Tool program (BLAST®, NCBI home page http://www.ncbi.nlm.nih.gov/blast) (Accessed last time: 08.12. 2005) and BLASTP from database Oralpro (Los Alamos Oral Pathogens Database http://www.oralgen.lanl.gov/) (Accessed last time: 08.12. 2005).
The first step PCR run used universal prokaryotic primers 785 and 422 in order to amplify the intergenic spacer region (2349bp). The PCR mixture was prepared for a 25µl reaction and it contained the following:
- HotStarTaq Master Mix 12.5 µl
- Forward Primer 1.25 µl
- Reverse Primer 1.25 µl
- Template DNA 4 µl
- H_2O 6 µl

The concentration of primers was 0.5µM.
The thermal cycler program started with an initial heat activation step at 95ºC for 15 minutes and ended with a final extension step at 72ºC for 10 minutes.

Amplification was performed using 27 cycles with the following temperature profile: 95ºC for 1 minute, 42ºC for 1 minute and 72ºC for 3 minutes.

The second PCR run used one specific primer AS2 and a universal prokaryotic primer 241 and produced one amplification fragment of 926bp.

The PCR mixture was prepared for a 25µl reaction and it contained:

- HotStarTaq Master Mix 12.5 µl
- Forward Primer 1.25 µl
- Reverse Primer 1.25 µl
- Template DNA 2 µl (first PCR products)
- H_2O 8 µl

The thermal cycler program started with an initial heat activation step at 95ºC for 15 minutes and ended with a final extension step at 72ºC for 10 minutes.

Amplification was performed using 21 cycles with the following temperature profile: 95ºC for 1 minute, 58ºC for 1 minute and 72ºC for 2 minutes.

All PCR runs included two non-template DNA samples as negative controls.

PCR products were analyzed by 2% agarose gel electrophoresis performed at 100V in 1x TAE buffer tris-acetate-EDTA pH 8.5. The protocol was the same as that used by Lamell et al. (2000) (57) and Leys et al.(1994) (45)with modification of amplification cycles as shown above.

Detection of *P. gingivalis* and identification of different *P.g.* strains

DNA isolated from the samples was analyzed for the presence of *P.g.* using a nested, two-step PCR procedure with two pairs of primers; first universal primers and second one specific and one universal primer. The amplification fragment contains the ribosomal intergenic space that can be used for determination of variability among different strains as shown below (Figure 6).

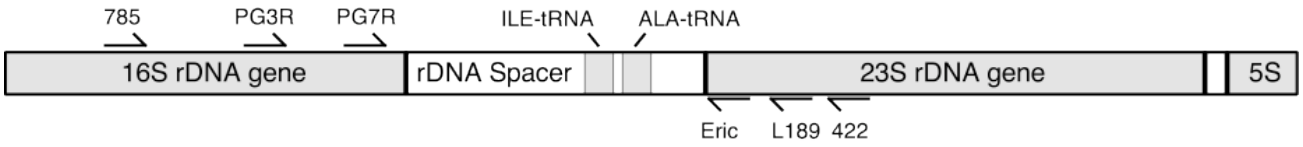


Fig 6. *P. gingivalis* ribosomal intergenic space and the primers positions Rumpf et al. (1999) (58).

The complete sequence of 16S-23S intergenic spacer region was obtained from GeneBank (ascension numbers AF118635, AF118634 and AF118633). Primers were checked with web Basic Local Alignment Search Tool program (BLAST®, NCBI home page http://www.ncbi.nlm.nih.gov/blast) (Accessed last time: 08.12. 2005) and BLASTP from database Oralpro (Los Alamos Oral Pathogens Database http://www.oralgen.lanl.gov/) (Accessed last time: 08.12. 2005).
In order to detect the strain differences, heteroduplex mobility analysis was used.
The PCR conditions were as follows:
The thermal cycler program started with an initial heat activation step at 95ºC for 15 minutes and ended with a final extension step at 72ºC for 10 minutes for all PCR steps.

1. First PCR run was carried using 4µl of DNA and universal prokaryotic primers 785 and 422. The running consisted of 27cycles of 92ºC for 1 minute, 42ºC for 1 minute and 72ºC for 3 minutes and produced an amplification fragment of 1983bp. The PCR mixture was prepared for a 25µl reaction and it contained:
- HotStarTaq Master Mix 12.5 µl
- Forward Primer 1.25 µl
- Reverse Primer 1.25 µl
- Template DNA 4 µl
- H_2O 8 µl

2. The second PCR was carried out using 2µl of products from the first amplification and primers PG3R and L189. The running consisted in 27 cycles of 92ºC for 1 minute, 55ºC for 1 minute and 72ºC for 2 minutes and the amplified fragment consisted in 1659bp.

The PCR mixture was prepared for a 25µL reaction and it contained the following:
- HotStarTaq Master Mix 12.5 µl
- Forward Primer 1.25 µl
- Reverse Primer 1.25 µl
- Template DNA 2µl
- H_2O 8µl

After the second run the PCR products were analyzed by 2% agarose gel electrophoresis performed at 100V in 1x TAE buffer tris-acetate-EDTA pH 8.5.

3. First PCR products from positive samples were amplified using primers PG7 and EricM. Two microliters of PCR products were used and the conditions were the following: 21cycles of 92ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute.

The amplified fragment length was 893bp.

The PCR mixture was prepared for a 25µl reaction and it contained:

- HotStarTaq Master Mix 12.5 µl
- Forward Primer 1.25 µl
- Reverse Primer 1.25 µl
- Template DNA 2 µl
- H_2O 8 µl

The PG3R and PG7R primers were the reverse sequence of two oligodeoxynucleotide probes that were describe in a previous article. As reference *P. gingivalis* W50 (ATCC 53978) was used for heteroduplex. Heteroduplexes were formed mixing 5µl of PCR product from *P. gingivalis* W50, 5µl of PCR product from clinical sample and 1µl of 10x annealing buffer (1M NaCl, 100mM Tris-HCl pH 7.8, 20mM EDTA). The DNA mixture was denatured at 95ºC for 5 minutes and then annealed by cooling to 25ºC at the rate of 1º per minute in a water bath. Samples were stored on ice until they are loaded on gels.

All PCR runs and heteroduplex forming steps included two non-template DNA samples as negative controls. Heteroduplexes were detected by polyacrylamide gel electrophoresis. Gels were run in a Bio-Rad apparatus at constant voltage (100V) for 210 minutes, stained by ethidium bromide 0.5µg/ml and visualized with UV light using the UVIprochemi system and gel analysis software Uvisoft (**UVItec Limited Cambridge CB4 1QB, United Kingdom)**. The protocol followed the one used by Leys et al. (1999) (46) with some modifications of PCR procedure and electrophoresis on acryl-amide gels as shown above.

To identify different strain types the heteroduplex patterns were compared with the migration pattern table available on internet *(P. gingivalis* strain W50 is combined with other 13 strains) (59). The picture that was used as reference for the heteroduplex patterns is shown in Figure 7.

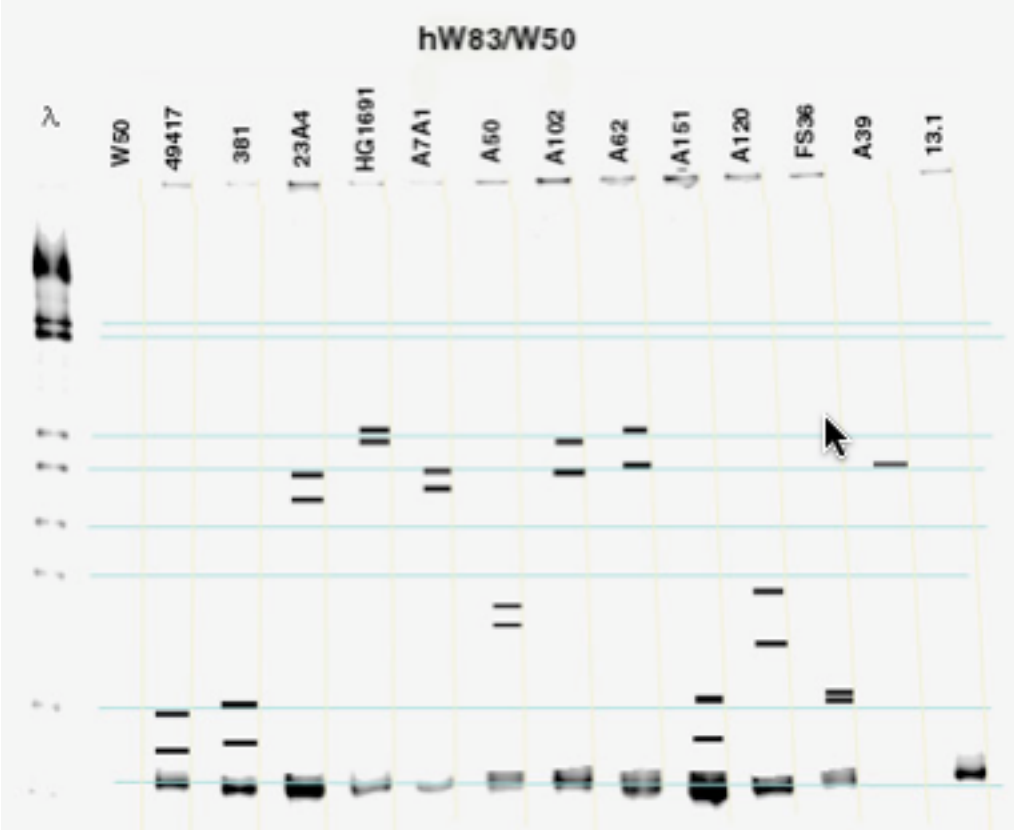


Fig 7. Heteroduplex migration patterns for W50 strain and other 13 different *P. gingivalis* strains available on Griffen, Leys and collaborators (http://www.dent.ohio-state.edu → Griffen → Lab homepage → Data → Heteroduplex patterns → Table) (Accessed last time: 08.12. 2005)

Detection of *T. denticola*

The PCR amplification used a *T.d.*-specific primer pair 16S rDNA (see page 21) and the amplification product consisted of one fragment of 316bp. The primers were described first in two papers that investigated putative periodontal pathogens and used in other articles that searched for *T.d.* in endodontic infections (39, 55). Complete sequence of 16S ribosomal RNA was obtained from GeneBank (accession number D85438). The primers were checked with web Basic Local Alignment Search Tool program (BLAST®, NCBI home page http://www.ncbi.nlm.nih.gov/blast) (Accessed last time: 08.12. 2005) and BLASTP from the database Oralpro (Los Alamos Oral Pathogens Database home page http://www.oralgen.lanl.gov/) (Accessed last time: 08.12. 2005).

The PCR mixture was prepared for a 25µl reaction and it contained:

- HotStarTaq Master Mix 12.5 µl
- Forward Primer 1.25 µl
- Reverse Primer 1.25 µl
- Template DNA 2 µl
- H_2O 8 µl

The thermal cycler program started with an initial heat activation step at 95ºC for 15 minutes and ended with a final extension step at 72ºC for 10 minutes. Amplification was performed using 36 cycles with the following temperature profile: 95ºC for 1 minute, 60ºC for 1 minute and 72ºC for 2 minutes. PCR products were analyzed by 2% agarose gel electrophoresis performed at 100V in 1x TAE buffer tris-acetate-EDTA pH 8.5.

All PCR runs included two non-template DNA samples as negative controls.

A 100bp DNA ladder digest served as the molecular weight marker.

The protocol was published by Siqueira et al. (2000) paper (55).

Detection of *P. intermedia* and *T. forsythensis*

A one step multiplex PCR that used one universal 16S rDNA forward primer and two specific reverse primers was carried out in order to identify the two bacteria in the same reaction. The PCR mixture was prepared for a 25µl reaction and it contained the following:

- HotStarTaq Master Mix 12.5 µl
- Forward Primer 2 µl
- Reverse Primer *Prevotella* 2 µl
- Reverse Primer *Tannerella* 2 µl
- Template DNA 2 µl
- $MgCl_2$ 25mM 1.5 µl
- H_2O 3 µl

The final concentration of $MgCl_2$ was 3mM.

The thermal cycler program started with an initial heat activation step at 95ºC for 15 minutes and ended with a final extension step at 72ºC for 10 minutes.

Amplification was performed using 30 cycles with the following temperature profile: 95ºC for 1 minute, 55ºC for 1 minute and 72ºC for 2.5 minutes.

Amplification products were separated on a 2% agarose gel in 1x TAE buffer tris-acetate-EDTA pH 8.5. The amplicons sizes of 663bp *for P.i.* and 844bp for *T.f.* were visualized using UVIprochemi system and gel analysis software Uvisoft **(UVItec Limited Cambridge CB4 1QB, United Kingdom)**.

All PCR runs included two non-template DNA samples as negative controls.

The protocol followed the Conrads et al. article (1999) (40) with some changes concerning the temperature profile and $MgCl_2$ concentration that were taken from Henegariu et al. article (1997) (60) and Qiagen Multiplex PCR Handbook (61).

Real time PCR

A real-time PCR procedure was used for relative quantification of *P. gingivalis* and *A. actinomycetemcomitans*. Quantification was performed using specific target sequences with double fluorescence labelled probes FAM and TAMRA and one universal 16S rRNA primer pair labelled with Yakima Yellow and dark quencher. Complete sequences of leukotoxin C gene of *A.a.* and Arg-gingipain gene of *P.g.* were obtained from GeneBank (accesion number U51862 and D64081, respectively).

The primers were used in three articles for real-time absolute and relative quantification (48, 62, 63). They were checked with web Basic Local Alignment Search Tool program (BLAST®, NCBI home page http://www.ncbi.nlm.nih.gov/blast) (Accessed last time: 08.12. 2005) and BLASTP from the database Oralpro (Los Alamos Oral Pathogens Database http://www.oralgen.lanl.gov/) (Accessed last time: 08.12. 2005).

The PCR mix was designed for 25µl reaction and it consisted of:

- TaqMan Universal PCR Master Mix 12.5µl
- Forward primer 2µl
- Reverse primer 2µl
- TaqMan probe 1µl
- DNA template 5µl
- H_2O 2.5µl

The final concentration of the primers was 400 mM and for the probes 200 mM.

The thermal cycling conditions were:
- 95ºC for 10minutes for AmpliTaq Gold Activation and
- 40 cycles consisting in 95ºC for 15 seconds and 60ºC for 1 minute.

The two most commonly used methods to analyze data from real-time, quantitative PCR runs are absolute and relative quantification. Absolute quantification determines the input copy number by relating the PCR signal to a standard curve.

Relative quantification is a simpler approach. It demands one target and one endogenous control gene (64). The resulting ratio it will report the difference between target and endogenous control for every sample to one chosen sample that can be used as a calibrator. The ΔΔCt method was used.

Absolute quantification

Using the same primers and probes as for relative run, absolute quantification was run.
All the calculations were done using the assumption that all oral bacteria have the same genome weight as *P.g.* that has been completely sequenced and for which the exact genome size (2.2Mb) and weight (2.37femtog 10^{-15}g) are known (65, 66). The total DNA concentration was obtained using GeneQuant spectrophotometer. The total number of copies was calculated using the genome weight.
Using the genomic DNA extracted from the laboratory strain cultures (*P.g.* ATCC 33277 and *A.a.* ATCC 33384) serial 5- and 10-fold dilutions that covered 5 logs were constructed. The *P.g.* serial dilution was used both for total bacteria and P.g counting. The standards were run in triplicate.

Data analysis

Counts of total bacteria identified by culture were calculated by direct counting of the selected colonies with regard to the original sample. Results were expressed in colony-forming units/sample (CFU/sample) and they were logarithmically transformed to improve the normality.
Counts of the selected pathogens identified by real-time PCR results were expressed in Ct that represents the cycle number at which the reaction begins to be exponential for a known

number of DNA copies (relative quantification). Absolute quantification provided number of total bacteria, *A.a.* and *P.g.*

The Spearman's correlation coefficient was calculated to assess the degree of correlation between CFU counts and real-time PCR results.

The Wilcoxon signed rank test (for two related samples) was used to compare combined periodontal and simple periodontal samples regarding the number of bateria assessed by culture and real-time PCR.

The McNemar test was used to test the differences of frequencies of positive samples for the five pathogens investigated.

The Spearman's correlation coefficient was used to calculate the correlation between total bacteria number in combined lesion periodontal vs. endodontic.

The kappa test was used to determine the level of agreement in bacterial detection between conventional PCR and real-time PCR.

The chi-square test was used to compare the association between different bacteria.

The STATA software (**Intercooled Stata 8.0 for Windows STATA Corporation USA**) was used to perform the analysis. P values < 0.05 were considered statistically significant.

Methodological consideration

Clinical consideration

The periodontal pocket depths were not recorded because the sample unit was considered the paper point that was used for sample collection both in periodontal pockets and root canals for a standard period of time. Correlating the results from periodontal and endodontic samples with respect to amounts of total bacteria, *P.g.* and *A.a.* was not considered relevant, taking into account particular features of the two tissues.

For 12 patients, previous medical records were not available. Any previous use or misuse of antibiotics within the previous three months could not be well documented.

Sample collection

The samples were collected using paper points and frozen at -70ºC within 1 hour after sampling. Collection lasted almost 3 months and the time from the sampling procedure until the processing of the DNA ranged between 3 and $3^1/_2$ months. One recently published article

showed modification of qualitative and quantitative results for total DNA, bacterial complexes and individual pathogens belonging to the "red complex" group of bacteria. The results indicate the persistence of "red complex" bacteria compared with other bacteria during longer storing periods but no modification within the group itself. However, the storing conditions were different (+4ºC and -20ºC and the period from 6 weeks up to 12 months) (67) from those I used.

Heteroduplex analysis

Heteroduplex mobility analysis is a fast and inexpensive method for determining relatedness between DNA sequences. This analysis is based on the observation that the structural deformations in double-stranded DNA that result from mismatches and nucleotide insertions or deletions cause a reduction in the electrophoretic mobility of these fragments in poliacrlyamide gels (68). The heteroduplexes are formed by the deliberate mixing of separately amplified reactions (mixture of PCR-amplified DNA fragments from divergent but related genes). When these products are separated on polyacrylamide gels, nearly comigrating homoduplex bands plus two additional slower migrating heteroduplex bands are observed (69).

The heteroduplex method proposed by Leys et al. (1999) for identification of different strains of *P.g.*, seems to be a reliable assay that avoids the need for cultivation (46). I used DNA isolated from pure culture of *P.g.* (W50 strain) to construct the duplexes and I got less clear bands than in the original paper. Leys et al. used intergenic space region fragment cloned into a plasmid and transformed into *Escherichia coli*.

Real-time relative quantification

There are two methods for relative quantification.

1. The relative standard curve method uses relative quantity expression to some basis sample, such as the calibrator. For all the samples, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1x sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. Standard curves are constructed using several dilutions for target and endogenous genes. Using the standard curves and C_t values for all the samples that result from the PCR running, the input amount target and endogenous control are obtained.

For every sample the target is normalized by dividing it to the endogenous control. One sample is designated as calibrator and all the normalized values are referred to it (70). There are other mathematical models for calculation of the ratio (71).

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct\ target(calibrator-sample)}}{(E_{end.control})^{\Delta Ct\ end.control(calibrator-sample)}}$$

2. The comparative C_t method uses an arithmetic formula rather than a curve. The amount of target, normalized to an endogenous control and relative to a calibrator is given by the formula: $2^{-\Delta\Delta Ct}$ where

ΔCt= Ct Target- Ct Endogenous control and ΔΔCt= ΔCt Sample- ΔCt Calibrator

It is presumed that the efficiencies of the target and the endogenous control are optimal and equal to 2 (71). Usually the efficiencies approach 2 when the amplicons range from 50bp to 150 bp (72). It is important to remember that the use of the ΔΔCt method requires a validation experiment in order to demonstrate that the efficiencies of target and reference are approximately equal. The absolute value of the slope of the log input amount versus Ct should be less than 0.1. If the efficiencies of the two systems are not equal, relative quantitation using standard curves should be performed (73).

For the relative quantification universal 16S rRNA gene was designated as endogenous control and leukotoxinC gene of *A.a.* and Arg-gingipain gene of *P.g.* were designated as target. Validation experiment used one clinical sample that was detected as positive for both *P.g.* and *A.a.* A dilution series of different input amounts that covered 5 logs were performed. The Ct values obtained after running a relative quantification were exported and analysed using Excel. Using the Excel "linest" function it was calculated the regression line for both the target genes and endogenous control gene (slope, intercept and Rsquared).

The efficiencies of PCR were calculated using the following formula:

$$\text{Efficiency} = 10^{-1/slope} - 1$$

Efficiencies of different PCR primers were compared and a relative efficiency function the input amount vs. ΔCt. was calculated as shown in Table 1.

The graphical representations of the functions are shown in Figure 8 and 9.

The following criteria were used:

- The slope of input amount versus ΔCt should be < 0.1.

- The differences in the primer efficiency between the gene of interest and endogenous control should not exceed 5% (70).

DNA ng	LogDNA	CtUni	CtPg	CtAa	EfficencyUni	Efficency Pg	Eficency Aa
200	2,30103	17,297	19,404	21,39	2,75903351	2,548583309	2,593047664
20	1,30103	19,852	21,976	23,872			
2	0,30103	20,988	23,012	25,208	Slope Uni	Slope Pg	Slope Aa
0,2	-0,69897	22,857	25,212	27,143	-1,7389	-1,818	-1,8003
0,02	-1,69897	24,489	26,876	28,756			
			DeltaCt Pg/Uni	DeltaCt Aa/Uni			
			2,107	4,093			
			2,124	4,02			
			2,024	4,22			
			2,355	4,286			
			2,387	4,267			
		Slope	-0,0791	-0,0614			

Table 1. Ct values and calculation of efficiencies obtained in the validation experiment.

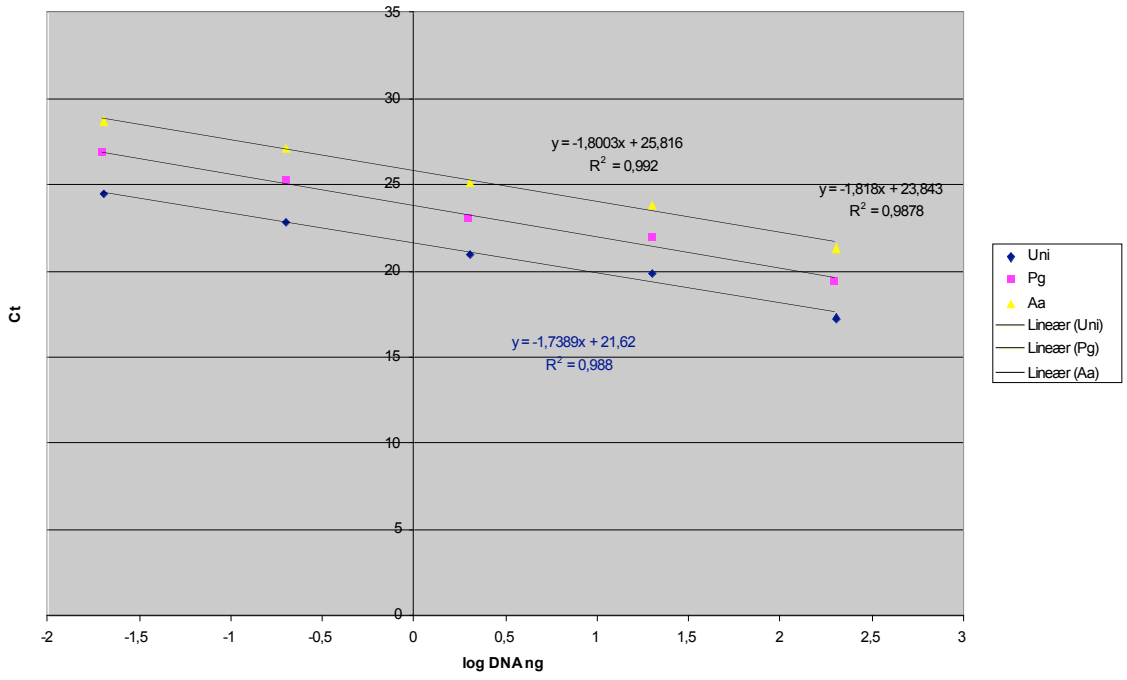


Fig 8. Standard curves for the three pairs of primers.

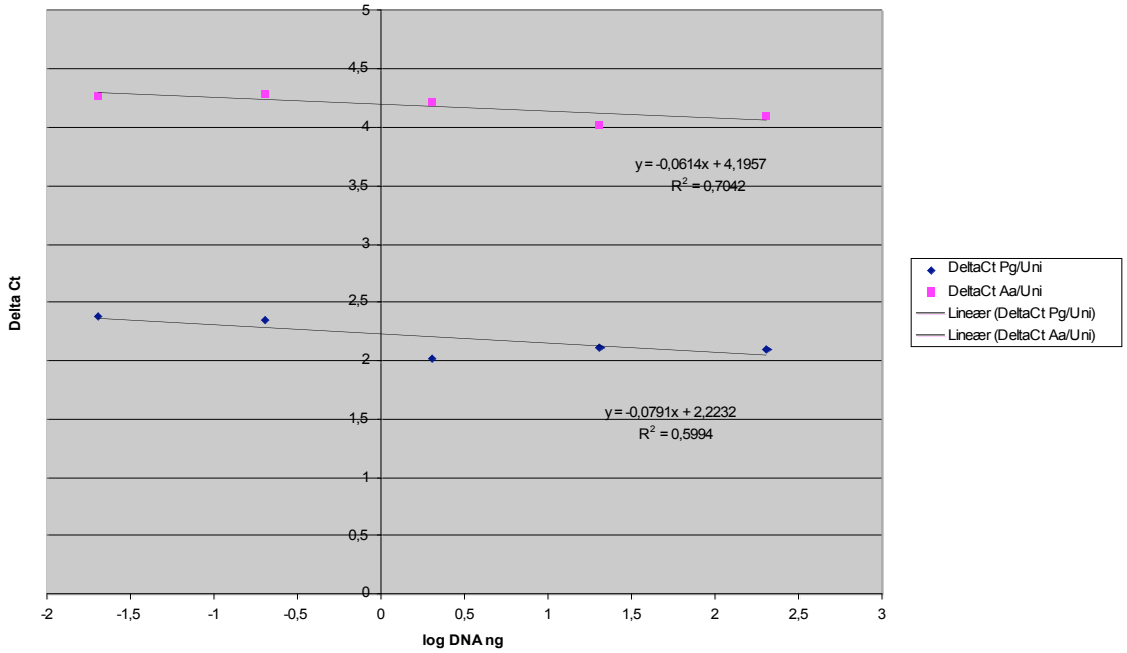


Fig 9. Delta Ct for the two targets *P.g.* and *A.a.* against endogenous universal 16S rRNA.

These requirements were fulfilled and the ΔΔCt method could be used.

Real-time absolute quantification

I used an absolute quantitation protocol to asses the copy number for total bacteria, *A. actinomycetemcomitans* and *P. gingivalis*.

Absolute quantitation of bacteria requires an exact standard curve that should cover 7 logs (from 10^1 to 10^7 copy number). The calibration curves can be based on known concentration of DNA standard molecules such as recombinant plasmid DNA or genomic DNA.

Because of the high cost and time-consuming procedure of producing plasmid DNA, I chose to use genomic DNA from *P. gingivalis* as source of DNA. I also used the assumption that the genome weights and 16S rRNA gene copy number employed for total bacteria quantification are not significantly different among oral bacteria (74, 75).

Using the concentration of DNA values and the genome weight of *P. gingivalis* 2.37 fg (10^{-15}g) (66, 76) I calculated the number of copies and created a standard curve.

I encountered problems in running these standard curves because the real-time PCR method was linear for samples containing more than $5x10^3$ copies see Figure 10.

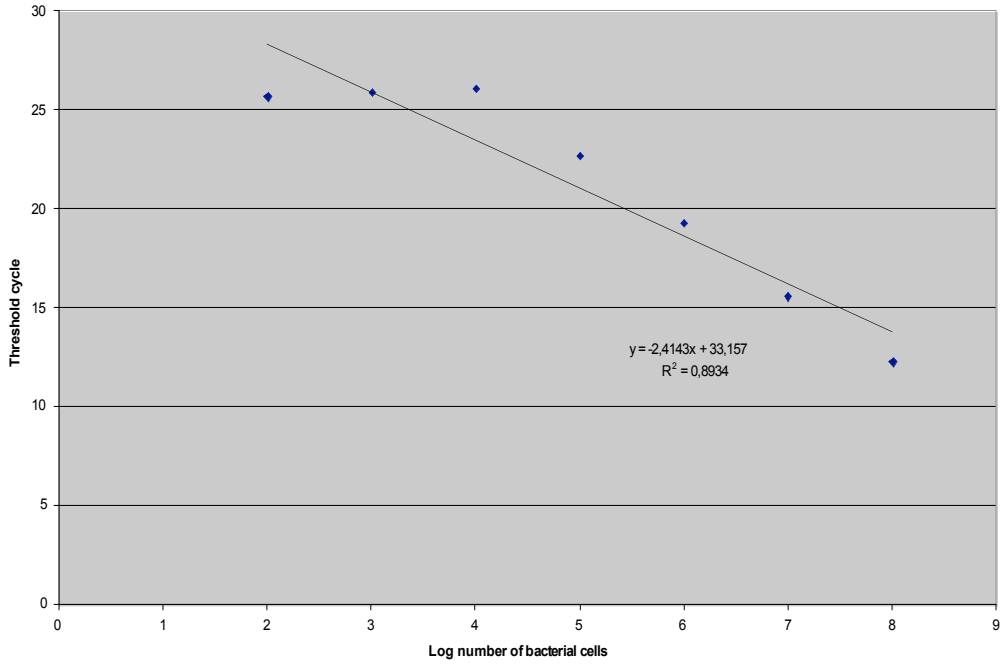


Fig 10. Standard curve generated for total bacteria quantification using genomic *P. gingivalis* DNA.

Similar studies that used real-time PCR to quantify periodontal bacteria managed to obtain a detection limit as low as 10^2 copies (77, 78). Socranski et al. (1994) developed a checkerboard DNA-DNA hybridisation assay for the detection of oral bacteria that has a cut-off value of 10^3 that proved to be useful for large-scale microbiological studies. It has not been regularly used for clinical diagnostic purposes (79).

Comparison of different laboratory methods

Culture and real-time quantification, conventional and real-time PCR identification of bacteria demonstrated fair to good agreement between the results and were in the range of previous reported studies (80).

RESULTS

Total anaerobic counts

Fifty of the 52 samples showed bacterial growth while two were culture negative (one endodontic and one simple periodontal sample). Table 2 shows that the mean CFU counts of the three types of samples were within the same log range. However, when comparing the median CFU counts, subgingival plaque from the combined lesions demonstrated a significant higher (p=0.048, Wilcoxon signed ranks test) median than did subgingival plaque from the simple periodontal lesions. There was a significant correlation between total anaerobic counts of paired periodontal and endodontic samples (Spearman's rho=0.7556 p=0.0002).

Table 2. Total anaerobic counts (CFU/ sample) and median, based on the mean of triplicate analysis of each sample, of subgingival and endodontic samples from combined periodontal-endodontic lesions and of subgingival plaque from simple periodontal lesions.

Anaerobic blood agar	Combined lesions		Simple periodontal lesions (n=13)
	Periodontal (n=19)	Endodontic (n=18)	
Counts	$3.96x10^5$-$3.98x10^7$	$5.62x10^5$-$2.67x10^7$	$2.7x10^5$-$3.27x10^7$
Median	$3.596x10^6$	$2.181x10^6$	$2.065x10^6$

Numbers of bacterial cells

Real-time absolute quantification

The amplification was linear for $\geq 5x10^3$ copies of total bacteria and the number of copies was calculated assuming that all oral bacteria have the genome mass equal to 2.37 fg femtogram=10^{-15}g (i.e. the genomic mass for *P.g.*) (66). The results of absolute quantification of total bacteria, *P.g.* and *A.a.*, respectively, determined in individual PCR runs, are presented in Table 3 as the range and median values.

Figure 11 shows total bacteria quantification and a comparison among the three types of samples. The highest median and widest range of bacteria were calculated for the combined periodontal samples.

Table 3. Number of copies of total bacteria, *P. gingivalis* and *A. actinomycetemcomitans* assesed by real-time PCR absolute quantification.

	Combined lesions		Simple periodontal (n= 14)
	Periodontal (n=19)	Endodontic (n=19)	
Total bacteria	$1.36x10^{7}$-$2.59x10^{14}$ $5.4x10^{9}$	$2.13x10^{5}$- $6.58x10^{11}$ $3.25x10^{9}$	$7.29x10^{6}$- $2.78x10^{12}$ $1.93x10^{9}$
P. gingivalis	0- $2.35x10^{10}$ $2.18x10^{7}$	0- $7.52x10^{8}$ 0	0- $1.82x10^{7}$ $9.47x10^{5}$
A.actinomycetem-comitans	0- $4.64x10^{7}$ $7.21x10^{4}$	0- $3.22x10^{5}$ 0	0-$8.94x10^{6}$ 0

A significant difference was found between the total number of bacteria in combined and simple periodontal samples (p=0.0258 Wilcoxon signed rank test) with more bacteria in combined ones. After normalization using logarithmic transformation, the t test confirmed this result (p=0.0154). Correlation testing of paired periodontal and endodontic samples showed significant correlation (rho=0.6659, p=0.0093).

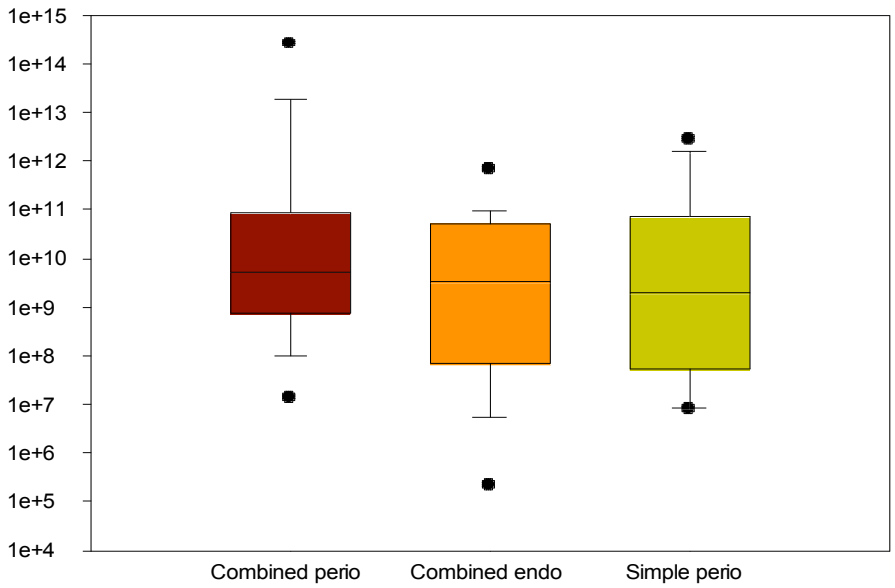


Fig. 11. Box plots showing the log copy numbers, range, median and percentiles (25% and 75%) of total bacteria.

A weak correlation between the total number of bacteria assessed by culture and real-time PCR was demonstrated (rho=0.275, p=0.0485).

A comparison between copy numbers of *P.g.* and *A.a.* is shown in Figure 12. Significantly higher numbers of *P.g.* was found in the combined periodontal samples compared with the simple periodontal ones (p=0.041 Wilcoxon signed rank test).

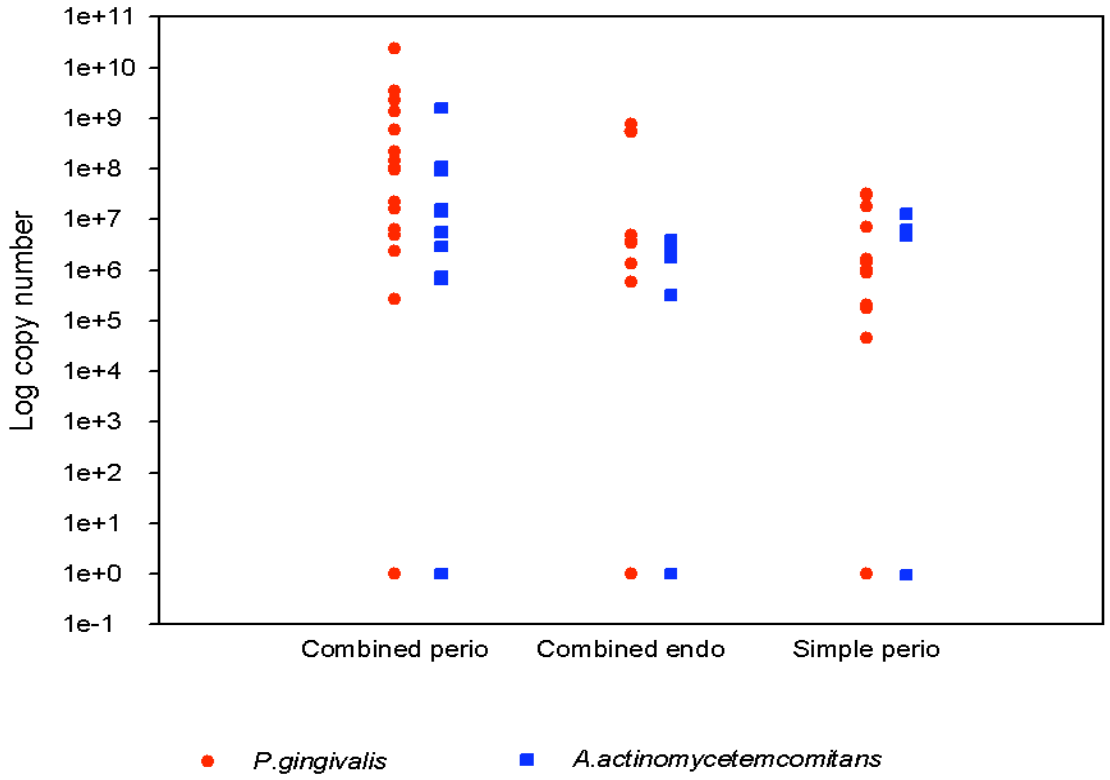


Fig. 12. Number of *P. gingivalis* and *A. actinomycetemcomitans* cells in samples from the three types of lesions determined by real-time PCR quantification.

Using the results from the absolute quantification the proportions *P.g.* and *A.a.* in the total bacteria were calculated. The range is shown in Table 4.

Table 4. Proportions of *P. gingivalis* and *A. actinomycetemcomitans.*

	% *P. gingivalis*	% *A. actinomycetecomitans*
Proportion	0 – 5.32	0 - 1.2

There were no significant differences between the proportions of *P.g.* and *A.a.* between the combined and simple periodontal lesion. (p=0.753 for *P.g.* and p=0.6 for *A.a.*, Wilcoxon signed rank test).

Real-time relative quantification

The results from the comparative ΔΔCt method were used to calculate the relative gene expression for *P.g.* and *A.a.*; the endogenous control gene was 16S rRNA for total bacteria and one sample served as the calibrator. Table 5 shows the ratio for the two bacteria.

Table 5. The relative ratio for *P. gingivalis* and *A. actinomycetemcomitans* expressed as the range and the median.

	P.g. relative to total bacteria	***A.a.*** relative to total bacteria
Ratio range	$4.9x10^{-2}$ – 33.2596	$8.46x10^{-3}$- 11.70
Ratio median	0.55869	0.2189

Detection frequencies of the species

After the second run of the nested PCR, the bands were visualised (Figures 13 and 14)

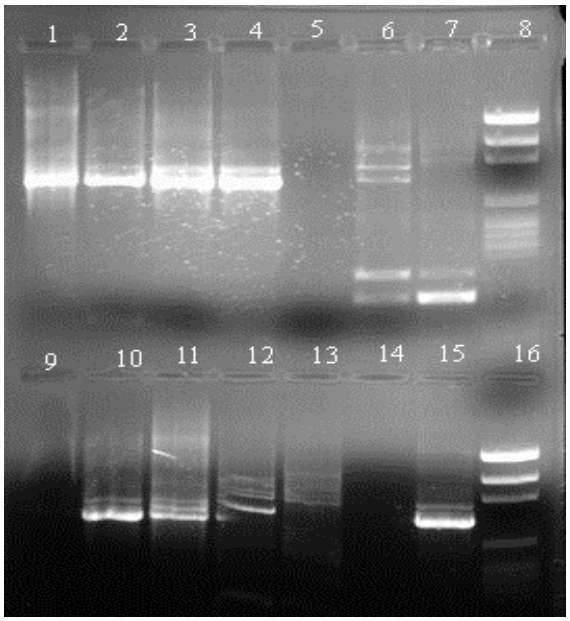


Fig. 13. Second amplification shows the 926 bp fragment obtained for identification of *A. actinomycetemcomitans*
Lanes 1-4: pooled positive samples
Lane 5: negative control
Lanes 6 and 7: non specific fragment
Lane 8: DNA marker
Lanes 9 and 14: negative sample
Lanes 10-12 and 15: positive samples

A.a. was detected in 57.89%, 21.05% and 42.86% of the combined periodontal, endodontic and simple periodontal samples, respectively.

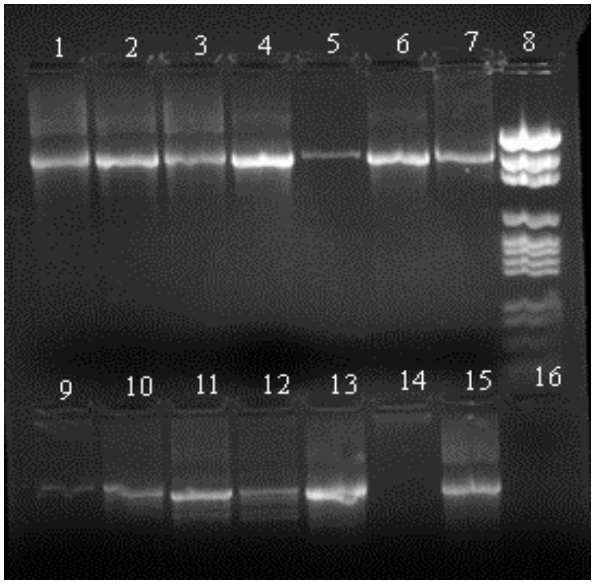


Fig. 14. 1659 bp fragment obtained after the second PCR.
Lanes 1-7, 9-13: positive pooled *P. gingivalis* samples
Lane 8: DNA marker
Lane 16: negative control

Similarly *P.g.* was identified in 78.95%, 42.11% and 78.57% of the samples.

Figure 15 shows the simultaneous identification of samples positive tested for *T.f.* and *P.i.* using a multiplex PCR procedure.

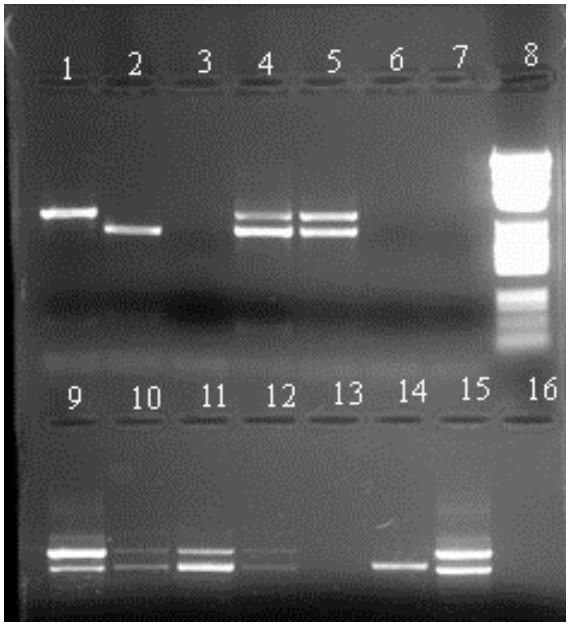


Fig. 15. Multiplex PCR showed 2 amplicons 663 bp for *P. intermedia* and 844 bp for *T. forsythensis*
Lanes 1,4,5, 9-12, 14 and 15: positive pooled *T. forsythensis* samples
Lanes 2,4,5,9-12 and 15: positive pooled *P. intermedia* samples
Lane 7: negative control
Lane 8: DNA marker

Conventional PCR showed 42.11%, 21.05% and 35.71% of samples positive for *P.i.*, 68.42%, 21.05% and 64.29% positive for *T.f.* and 47.37%, 26.32% and 35.71% positive for *T.d.* in the three types of lesions.

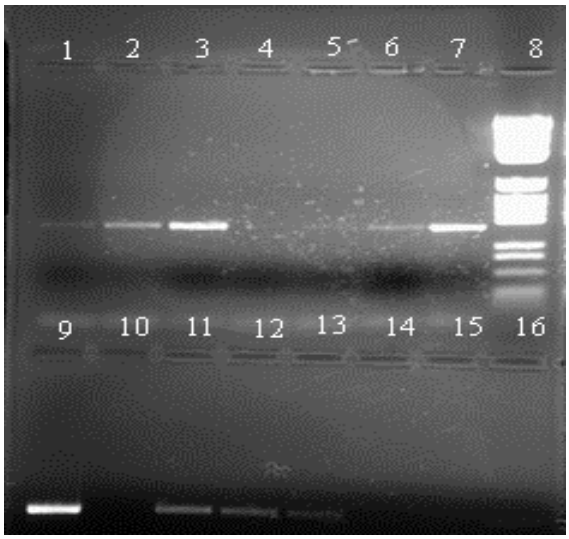


Fig. 16. Positive samples for *T. denticola* showing a 316 bp amplicon
Lanes 1-3, 6, 7. 9 and 11-13: positive *T. denticola* samples
Lane 14: negative control
Lane 8: DNA marker

The results from conventional PCR identification are summarized in Table 6.

Table 6. Percentages of samples that tested positive using conventional PCR.

Bacteria	Combined lesions		Simple periodontal lesions (n= 14)
	Periodontal (n=19)	Endodontic (n=19)	
P. gingivalis	78,95	42,11	78,57
A. actinomycetemcomitans	57.89	21.05	42.86
T. forsythensis	68.42	21.05	64.29
P. intermedia	42.11	21.05	35.71
T .denticola	47.37	26.32	35.71

There were no significant differences between the detection frequencies of the five pathogens in the three types of lesions.
A significant association between *P.g.* and *T.d.* (p=0.033) and between *P.g.* and *T.f.* (p=0.035) was demonstrated only in the combined periodontal samples group.

Real-time PCR provided not only quantification of pathogens but also identification (Figure 17). The results obtained by conventional and real-time PCR were compared.

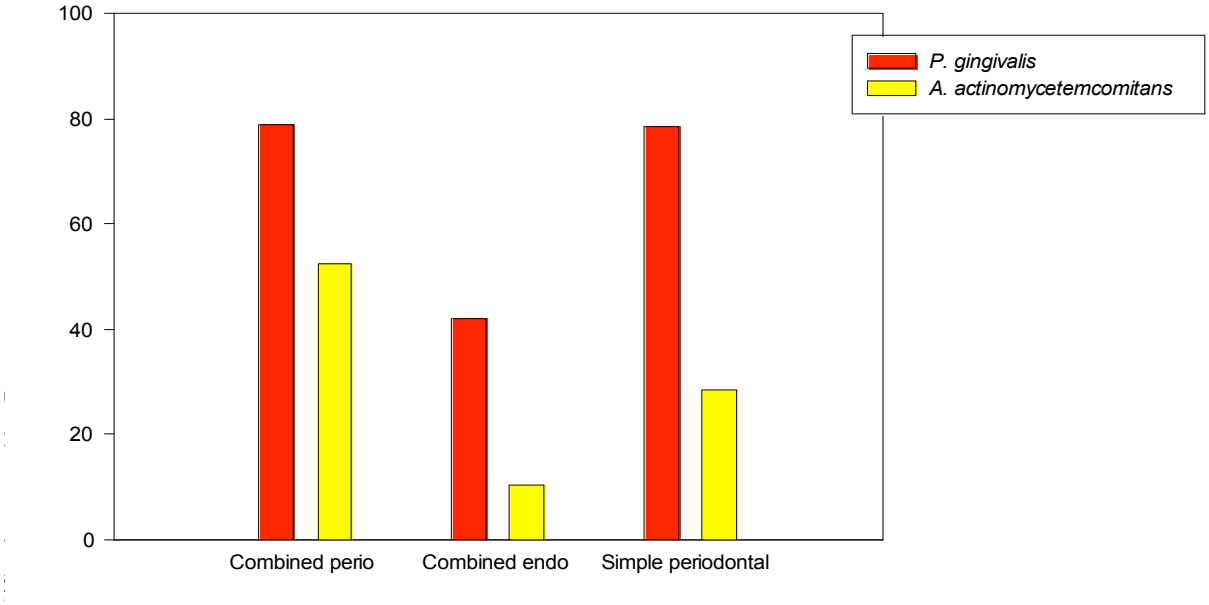


Fig. 17. Percentages of samples demonstrating *P. gingivalis* and *A. actinomycetemcomitans.*

The agreement between the conventional PCR and real-time PCR identification of the two species was 84.62% for *P.g.* (Kappa 0.6601 Std. error 0.1387 and p<0.0001) and 78.85% for *A .a.* (Kappa 0.5503 Std. error 0.1376 and p<0.0001). Table 7 shows the number of samples from the three types of lesions that tested positive for *P.g.* and *A.a.* with both detection methods.

Table 7. Number of samples tested positive for *P. gingivalis* and *A. actinomycetemcomitans* using conventional PCR (PCR) and real-time PCR (RT-PCR).

Bacteria	Combined lesions				Simple periodontal	
	Periodontal (n=19)		Endodontic (n=19)		(n=14)	
	PCR	RT-PCR	PCR	RT-PCR	PCR	RT-PCR
P. gingivalis	15	15	8	8	11	11
A. actinomycetem-comitans	10	10	4	2	6	4

P. gingivalis strains

The products from the second PCR were mixed and heteroduplexes that were formed were analyzed.

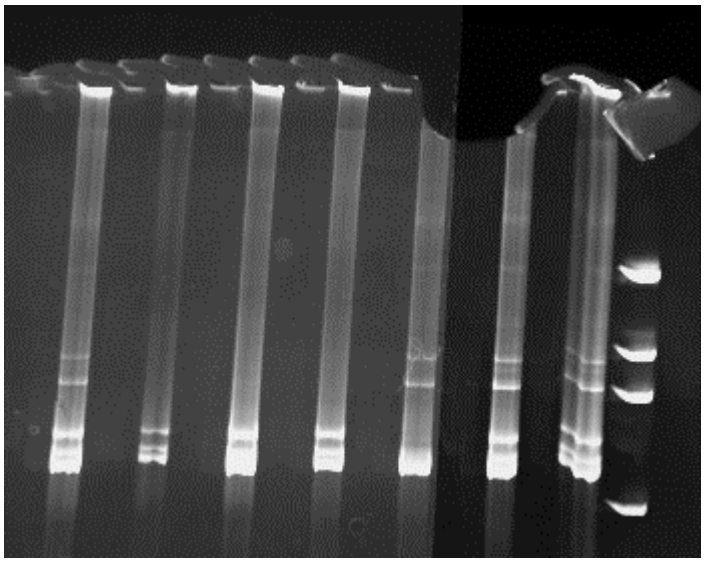


Fig. 18. Acrylamide gel showing formation of heteroduplexes between sample strains and W50 lab strain.

From all the samples 18 contained one strain, 14 showed two different strains and only two had three different strains; the distribution of strains in the samples is presented in Table 8. Six combined lesions contained the same type of strain/strains.

Table 8. Distribution of *P. gingivalis* strains in the three types of samples.

	Combined lesions		Simple periodontal (n=14)
	Periodontal (n=19)	Endodontic (n=19)	
Positive samples	15	8	11
One *P.g.* strain	8	4	6
Two *P.g.* strains	7	3	4
Three P.g strains	2	0	0

DISCUSSION

The scarcity of literature on this topic and the challenges in treating some of these cases prompted my thesis. The present study aimed at investigating the occurrence of some principal periodontal pathogens in combined perio-endo lesions. It is known that most periodontal pathogens are also endodontic pathogens (81). Because of lack of dental history it was not possible to subgroup the lesions according to Simon et al. (1972) classification (12). For practical reasons, the study focused only on five selected periodontal pathogens and single periodontal lesions were included as a reference group. The reason for this choice was determined by clinical and diagnostic problems; usually it is easier to find multiple periodontal lesions from the same patient and not so often multiple teeth with endodontic problems in the same patient.

Quantification

Culture was used for quantification of anaerobic bacteria that represent the main pathogens both in subgingival and root canal spaces (5). The number of bacteria ranged from $2.7x10^5$ to $3.98x10^7$ and these results correspond to those reported by Rupf et al. (2000) (29).
Real-time PCR using universal 16S rRNA primer and specific *P.g.* and *A.a.* primers allowed quantification of the total number of bacteria as well as of *P.g.* and *A.a.* My results are in accordance with previous reports of real-time quantification for total bacteria (82), for *P.g.* (80, 82) and for *A.a.* (80).
For both culture and real-time quantification there were statistical significant correlations between the total numbers of bacteria in combined periodontal and combined endodontic samples. The same was reported by Rupf et al. (2000) (29).
Culture and real-time counts showed also significantly higher numbers of bacteria in combined periodontal samples compared to the simple periodontal ones. To my knowledge such data have not been presented before. The higher number could be explained by bacterial migration between the root canal and the periodontium.
Absolute quantification for *P.g.* and *A.a.* made possible the calculation of their proportions in the total bacteria. My results showed percentages in the same range for *P.g.* as published elsewhere (80) and higher percentage values for *A.a.* than previously reported (63).

Relative quantification using the same real-time PCR reaction protocol and ΔΔCt calculation gave results about the relative gene expression of *P.g* and *A.a* against the universal 16S rRNA. The values obtained could not be compared because no such data seem to have been published.

PCR identification

Different PCR protocols were used for identification of the five periodontal pathogens. Identification of *A.a.* with both conventional PCR and real-time PCR showed similar detection frequencies of positive periodontal cases as previous reports (29). For the endodontic samples there was a difference in positive samples number. Conventional PCR demonstrated 21.1% positive samples (4 cases out of 19) while real-time PCR showed only 10.5% positive samples (2 cases out of 19). This difference can be explained by the different primers that were used. The real-time PCR primers were more specific as they amplified the leukotoxin C gene of *A.a.* The leukotoxin is assumed to enable *A.a.* to evade the main defence line of the periodontal pocket and to significantly contribute to the pathogenesis of periodontal disease. Even though all strains of *A.a.* harbour the leukotoxin gene, the production of leukotoxin varies highly between various strains (83, 84). This species is not a usual endodontic pathogen although it was detected in extraradicular infections (85) and in cases of intraradicular infections (86). In one case report about an endodontic infection caused by localized periodontitis, the checkerboard DNA-DNA hybridisation analysis did not disclose *A.a.* in the root canal although the adjacent periodontal pocket contained the pathogen (31).

Identification of *P.g.* showed similar percentages of positive samples as in previous studies on combined lesions (87). Using the heteroduplex analysis the samples showed from one to three different *P.g.* strains in my study. The same strains were present in six combined lesions. The results are in concordance with other findings about different *P.g.* strains in periodontal and endodontic samples (88).

P.i., *T.f.* and *T .d.* occurred in the same range of positive samples in periodontal and endodontic samples as reported by Rupf et al. (2000) (29).

No significant differences were found between the three types of samples; combined periodontal, endodontic and simple periodontal regarding the identification frequencies of these pathogens.

T.d. was identified in 26.3% of the endodontic samples. Previous studies discovered even a higher percentage of positive endodontic samples and gave reasons for possible pathogenesis of periradicular lesions (55).

Lately there were more studies on biofilms and bacterial complexes in subgingival plaque that wanted to increase our understanding of the periodontal disease processes (89).

The red complex of species (*P. gingivalis, T. forsythensis and T. denticola*) was found to be strongly associated with clinical signs of periodontitis. This complex was assessed also in endodontic infections in order to evaluate the possible participation of the red complex bacteria in pathogenesis in periradicular disease (90).

In the present study, I found significant associations between *P.g.* and *T.f.* and *P.g.* and *T.d.*, respectively but only in combined periodontal samples. These findings are supported by other studies that revealed frequent associations between these bacteria and thus a positive ecological relationship (91).

Perspectives

The topic of pathologic interactions between pulpal and periodontal tissues is still a “hot” one because of some unanswered questions and controversies mentioned in the introduction section of my thesis. The study of microbial complexes formation together with the immune host response can bring new valuable information about combined perio-endo lesions.

CONCLUSIONS

1. The following findings support the existence of bacterial migration through physiological pathways between the pulp and the periodontium:
   a) The significantly higher bacterial levels in subgingival plaque from combined periodontal-endodontic lesions than in corresponding samples from the autologous simple periodontal lesions.
   b) The significant positive correlation between the bacterial levels of subgingival and endodontic samples from combined periodontal-endodontic lesions.
   c) The presence of the same *P. gingivalis* strain(s) in paired periodontal and endodontic samples.
2. Associations of bacteria belonging to the so-called red microbial complex were detected only in combined periodontal samples.
3. Based on the clinical data available it was not possible to subgroup the 19 combined lesions.

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Appendix I

In house prepared fluid cultivation media

Tryptone Peptone	15 g
Sodium chloride NaCl	5 g
Potassium dihydrogen phosphate KH_2PO_4	1.5 g
Disodium hydrogen phosphate dehydrate $Na_2HPO_4 \times 2H_2O$	3.5 g
Ammonium sulphate $(NH_4)_2SO_4$	0.5 g
Sodium hydrogen carbonate $NaHCO_3$	0.5 g
Yeast extract	3.0 g
Ro-water	1000 ml

Anaerobic blood-agar

Tryptose agar	41 g
Yeast extract	3 g
Menadion	1 ml
Sheep blood	50 ml
Ro-water	1000 ml

Trypticase soy-bacitracin-vancomycin (TSBV) medium

Trypticase soy agar	40g
Yeast extract	1g
Horse serum	50 ml
Bacitracin	0.038 g
Vancomicin	0.0025 g
Ro-water	1000 ml

Appendix II

QIAmp DNA Mini Kit (QIAGEN)
Protocol for isolation of genomic DNA from bacteria

1. Pellet bacteria by centrifugation for 10 min at 5000 x g (7500rpm).

2. Suspend bacterial pellet in 180µl of the appropriate enzyme solution (20 mg/ml lysozyme or 200 µl/ml lysostaphin; 20 mM Tris-HCl, pH 8.0; 2mM EDTA; 1.2% Triton®).
3. Incubate for at least 30 min at 37ºC.
4. Add 30 µl Proteinase K and 200 µl Buffer AL. Mix by vortexing.
5. Incubate at 56º C for 30 min and then for a further15 min at 95º C.
6. Centrifuge for a few seconds.

7. Add 200 µl ethanol (96%-100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
8. Carefully apply the mixture from step 4 (including the precipitate) to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
9. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube and discard the collection tube containing the filtrate.
10. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
11. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.