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Identification of yeast and non-pigmented cultivable endodontic bacteria in adult Portuguese patients

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Introduction This study has focused on the identification of the yeasts and non-pigmented bacteria present on adult patients with necrosis or apical periodontitis and the ones who resisted chemomechanical preparation and intracanal dressing with calcium hydroxide paste (Ca(OH)₂) or 2% chlorhexidine digluconate gel (CHX).

Methods 69 single-rooted teeth of adult patients with necrosis associated or not with apical periodontitis were selected (strict inclusion criteria); CHX group: 34 teeth; Ca(OH)₂ group: 35 teeth. Bacteria samples were taken at baseline (S1), after chemo-mechanical preparation (S2) and after 14 days of intracanal dressing (S3). Bacteria and fungal presence was evaluate by means of culture in three atmospheres (aerobic, anaerobic, microaerophilic) in appropriate culture broths. Strict techniques were used for serial dilution, plating, incubation and identification.

Results The most represented, abundant and prevalent strains of non-pigmented bacteria were *Propionibacterium acnes* (detected in S1, S2 and S3), *Gemella morbillorum* and *Clostridium difficile*. *Candida albicans* was found in 9 patients. The higher number of isolates proceeded from S1, being S2 the moment with lowest number of isolates. CHX had a worst performance in disinfection of the root canal system; consequently the number of isolates from S3 samples was bigger compared to Ca(OH)₂. The number of identified bacterial species per canal/moment of sampling, varied from zero till 5, including yeasts (*Candida albicans*). **Conclusions:** Our findings confirm that the microbiota from primary endodontic infections is polymicrobial, and the anaerobes Gram-positive non-pigmented bacteria are well represented. CHX performed worse, consequently the number of isolates from S3 samples was bigger when compared to Ca(OH)₂ as well as with diagnosis of necrosis.

Keywords Endodontics, Culture, Root Canal Sampling

1. Introduction

Endodontic infections have been traditionally studied by Culture methods and over 460 bacterial taxa have been associated with infected root canals (Siqueira & Rôças 2009).

Several studies on root canal infections have focused on strict anaerobic bacteria due to their predominance in samples taken from untreated teeth with necrotic pulps (Sundqvist 1994, Le Goff et al. 1997, Dahlén et al. 2000, Gomes et al. 2004)

Knowing that diverse community is yet far away from being totally described and to our information there are not Portuguese data published on this matter, the purpose of this study was to identify before treatment whose species of non-pigmented bacteria and yeasts were in the selected infected root canals and also to compare the antimicrobial efficacy of calcium hydroxide paste (Ca(OH)₂) or 2% chlorhexidine digluconate gel (CHX) when used as intracanal dressings for 14 days.

2. Material and Methods

2.1. Subjects and Case Selection

Sixty nine patients who attended the Pedagogical Clinic of the Faculty of the Health Sciences of the University of Fernando Pessoa in Oporto for treatment of pulpar necrosis associated or not with apical periodontitis were included in this investigation.

The study was approved by the Ethics Committee of the Health Sciences Faculty of Fernando Pessoa University, and the patients understood and gave written informed protocol consent before the beginning of the study.

On the basis of stringent inclusion criteria (Siqueira et al. 2007), 69 single-canalled, with intact pulp chamber walls, necrotic pulps confirmed by negative response to sensitivity pulp tests, and in some cases clinical and radiographic evidence of asymptomatic apical periodontitis (AP) were integrated: 43 had AP and 26 had only pulp necrosis.

Canals were randomly divided into two sub-groups for standard root canal instrumentation and irrigation with 3% NaOCl followed by dressing with one of the following: 34 with calcium hydroxide paste (Ca(OH)₂) and 35 with 2% chlorhexidine digluconate gel (CHX).

Aseptic technique was always used.

2.2. First Session

S1 sample was acquired and transferred into vials containing 2 ml sterile Reduced Transport Fluid (RTF) as described (Siqueira et al. 2007), using 3% NaOCl and sterile cotton swabs, collected in tubes with 5ml Phosphate Buffer Saline. The working length (WL) was established with Root ZX apex locator (Morita, Korea, Japan) and confirmed by digital X-ray. Canals were enlarged with Protaper instruments (Dentsply Maillefer, Switzerland) according to manufacturer's instructions. Irrigation with 2 ml 3% NaOCl (27G needle) between consecutive files and #10 K-file 1mm beyond the WL were used to prevent apical blockage.

After smear layer removal (5 ml 10% Citric Acid, 5ml 3%NaOCl and 5ml 10% sodium thiosulfate (Na₂S₂O₃)), S2 sample was acquired. After drying, intracanal dressings were applied (Siqueira et al 2007). Access cavities were then sealed with Coltosol (Coltène/Whaledent Inc, USA) for 14 days.

Paper points used for sampling of the canals were transferred into vials containing Reduced Transport Fluid (RTF) to the laboratory within 2 hours (Siqueira et al. 2007).

2.3. Second Session

After similar field disinfection and sterility control sample and restoration removal (Siqueira et al 2007), medication was rinsed out with 5 ml of saline sterile solution for the Ca(OH)₂ group or mixture of lecithin, Tween 80 and Na₂S₂O₃ for the CHX group. S3 samples were then acquired and stored as above. Afterwards, canals were filled with gutta-percha and TopSeal (Dentsply, Maillefer, Ballaigues, Switzerland), sealed with Synergy D6 Flow (Coltène/Whaledent, USA) and the access cavity temporized.

3. Laboratory Procedures -Traditional Culture Identification

Samples were transported to the laboratory within two hours after collection for microbiologic processing.

Samples in RTF vials were vortexed for 30 seconds, and 10-fold serial dilutions to 10⁻³ (for S1 samples) or 10⁻² (for S2 and S3 samples) was made in PBS solution (Chu et al. 2006). Aliquots of 100 µL from the undiluted suspension and the highest dilution were each spread onto BHI agar plates and Anaerobe Basal Agar plates (Oxoid, United Kingdom) both supplemented with 5% defibrinated horse blood (Oxoid, United Kingdom). Plates were incubated within jars (Merck, Germany), at 37°C, anaerobically (85% N₂, 5% CO₂ and 10% H₂) with Anaerocult® A sachets (VWR, Merck Millipore, Germany) for 14 days and for 48 hours, aerobically and microaerophilically (in 10% CO₂) with Anaerocult® C sachets (VWR, Merck Millipore, Germany).

The same dilutions were plated onto Sabouraud–dextrose agar (Oxoid, United Kingdom), supplemented with 100 mg/ml chloramphenicol (Sigma-Aldrich USA), in order to detect yeasts.

After incubation, Colony-Forming Units (CFU's) were counted in each medium and different representative colonies were isolated. The isolation of non-pigmented bacteria and yeasts was performed using traditional culture procedures.

For biochemical identification, preliminary characterization of microbial species was based on colony features, cell morphology, Gram staining and catalase production. Bacterial identification was performed using the appropriate commercial biochemical test (API's system, bioMérieux): API 20Strep (BioMérieux SA, Marcy-l'Etoile, France) for Gram positive cocci, catalase negative (*Streptococci* and *Enterococcus*), as well as API Staph (BioMérieux SA, Marcy-l'Etoile, France)- a sensitive and specific test for Gram positive cocci catalase positive (clinical *Staphylococci* and *Micrococci*), and also RapID ANA II System (Innovative Diagnostic Systems Inc., Atlanta, GA)] for anaerobic bacteria, being cocci or bacilli Gram-positive or negative .

After the appropriate period of incubation (the reactions (enzyme profile) were read visually and the identification of the strains was obtained by referring to the API Analytical Profile Index (version 1.1 from BioMérieux SA) and by using a computer-generated code compendium supplied by the manufacturer in the case of Rapid ANA II. A presumptive identification of the isolates was made at the genus level and, whenever possible, at the species level.

Regarding to the presumptive identification of yeast inoculations of 2 types of media were used, after growth of isolates on the respective medium mentioned: a) CHROMagar Candida Medium (Becton Dickinson GmbH, Germany) being green colonies identified as *Candida albicans*; b) Half Biggy agar in which the brown coloured colonies indicated grow of *Candida albicans*.

4. Results

4.1. Microbiological bacterial diversity

All swab samples, took for sterility control, were negative, indicating a disinfection of the operation field.

Isolates were recovered from 34 patients at the 3 moments of sampling (S1, S2 and S3). The higher number of isolates proceeded from samples at baseline (S1), being S2 the moment the one with lowest number of isolates. At S1 57 of the 69 (82.6%) canals had cultivable bacterial growth and 11 samples had fungal presence. After intracanal dressings for 14 days (S3 samples), 35 canals, respectively 10 for the Ca(OH)₂- and 25 for the CHX-group showed growth.

The number of identified bacterial species per canal/moment of sampling, varied from zero till 5, including yeasts (*Candida albicans*). The most frequent number of species identified was one, and the maximum number of identified bacterial species/canal diminished in S2 (one) and S3 (two).

From 53 isolates, 42 were of non-pigmented bacteria that have been identified by their Gram-staining properties, morphology, and API identification Kit applied the anaerobic Gram-positive rod *Propionibacterium acnes* (Actinobacteria) was the most prevalent, being detected in S1, S2 and S3, followed by the facultative anaerobic, Gram-positive coccus *Gemella morbillorum* (Firmicutes) and by anaerobic Gram-positive bacteria *Clostridium difficile* (Firmicutes), *Actinomyces meyer* (Actinobacteria), *Bifidobacterium spp.* (Actinobacteria), *Micrococcus spp.* (Actinobacteria), *Peptostreptococcus anaerobius* (Firmicutes), *Peptostreptococcus prevotii* (Firmicutes), *Staphylococcus aureus* (Firmicutes) and *Streptococcus constellatus* (Firmicutes) were the ones which were detected in two samples. *Enterococcus faecalis* was detected in one sample at S1.

5. Comparison of the different intracanal dressings

The majority of species recovered in S1 were not detectable in the post-treatment samples and the majority of isolates belonged to cases of AP. In S2, occurred the fewer number of isolates, being *Micrococcus spp.* and *Propionibacterium* examples of them. CHX had a worst performance in disinfection of the root canal system; consequently their number of isolates (eight) from S3 samples was bigger when compared to Ca(OH)₂ (one) and with diagnosis of necrosis and included *Actinomyces israeli* (Actinobacteria) and *Clostridium innocuum* (Firmicutes) (obligate anaerobes rod Gram-positive), *Micrococcus spp.* (strictly aerobic cocci Gram-positive), *Propionibacterium acnes* (facultative anaerobe rod Gram-positive) and *Staphylococcus aureus* (facultative anaerobe cocci Gram-positive).

Of the six microorganisms that were observed in S3, three were not detected in the previous collections.

The presence of yeast *Candida albicans* was found in four isolates in S1, two in S2 and five in S3 equally numbered for necrosis and AP in S1 and S2, but more prevalent in S3 when the initial diagnosis was AP (four) compared with necrosis (one).

6. Discussion

Microorganisms were mostly recovered from S1, data in agreement with previous studies that showed the relationship between microorganisms and the development of apical periodontitis (Kakehashi et al. 1965, Fabricius et al. 1982, Le Goff et al. 1997, Dahlén et al. 2000, Lana et al. 2001). Moreover, this study proved the polymicrobial nature of the primary endodontic infections (necrosis associated or not with AP).

Despite mechanical instrumentation and disinfection of the root canal system, there were microorganisms detected in S2, clearly showing that root canal preparation is unable to eliminate all bacteria from the root canal system (Gomes et al. 1996a, Sydney 1996, Sjögren et al. 1997, Chavez et al. 2003). That's why it has been emphasized that antimicrobial agents should be used between appointments (Ando & Hoshino 1990, Oguntebi 1994, Siqueira et al. 1996, Chavez et al. 2003). Possible factors permitting microorganisms to survive endodontic procedures include the ability of bacteria to invade dentinal tubules (Oguntebi 1994), and secretion of metabolic products that may counteract the antibacterial effect of the endodontic treatment. And so, bacteria have the capacity to promote reinfection of the root canal after endodontic therapy.

It is important to emphasize that the samples were processed in the lab within 2 h to preserve the reproductive capacity of bacterial cells and to prevent the growth of microorganisms in the sample.

Bacteriological sampling procedures and Culture processing may not provide an accurate reflection of the root canal microbiota because many types of organisms fail to survive for identification under regular laboratory conditions.

In fact, growth of microorganisms in the lab has the advantage of allowing the identification of a large variety of species in the same sample, permit determination of antimicrobial susceptibility of isolated, as well as the study of physiology and pathogenicity. However, the identification based on phenotype features have limitations of diverse nature, namely: are costly, may take several days to weeks to identify fastidious anaerobes, have low sensitivity, specificity also tends to be low and it depends on the microbiologist' experience and, still, the means of transport are

laborious and time-consuming. On the other hand, do not allow culture of all species. Either because they are not present, in the midst of artificial culture, essential nutrients or growth promoters, either by toxicity of the own culture medium, either by the presence, in the middle, of injunctive substances produced by other species of the same sample, either by metabolic dependency of other species to grow (not present on the board of growth). Finally, if the bacteria are sleepers, they may not be able to split or form colonies in agar without previously resurrection. Another paradigm of this matter is the fact that, even if culture of bacteria is achieved, species can be difficult to identify. Therefore, Culture underestimates the identification of the endodontic pathogens being in approximately 50% of the oral microflora unculturable (Socransky et al. 1963, Pasteur et al. 2001). It is therefore probable that unculturable organisms are present in endodontic infections and may play a role in the pathogenesis of the lesions (Munson et al. 2002).

Probably, although there have been 36 pigmented colony isolates, (mostly belonging to S1 samples, being although approximately one third present at S3 – data not shown) its identification was not possible. In fact, the ones associated with endodontic infections, mostly belong to *Porphyromonas* and *Prevotella* species (Listgarten et al. 1993, Jousimies-Somer et al. 2002, Mosca et al. 2007) and are Gram-negative, bacilli. In some cases the identification process was not possible to be performed immediately after the incubation period of the pure cultures. So, they were frozen (at -20°C), fact that may have compromised their grow in order to identify them with the methodology adopted in our investigation based on Culture. Perhaps with the application of Molecular techniques it would be possible to confirm this suspicion.

The precautions taken in this study, such as sterile instruments, gloves and rubber dams, sterility controls before and after opening the pulp cavity, minimization of the time taken to Culture the samples and having a single practitioner performing the procedure, reduced the risk of contamination to a minimum but the probability of contamination cannot be dismissed (Le Goff et al. 1997, Molander et al. 1998).

The identity of the isolates of non-pigmented bacteria was consistent with that reported in previous studies, being dominated by anaerobes, particularly Gram-positive taxa of the phylum Actinobacteria and Firmicutes (Sundqvist, 1992, 1994; Saito et al. 2006).

Clostridium and *Actinomyces* species were the seconds most commonly isolated. This genus, which includes both obligate and facultative anaerobes, was less frequently isolated after endodontic procedures. However, it was noted, unlike *Gemella morbillorum*, *Propionibacterium acnes* (each one with 4 isolates of the total before treatment) had the higher prevalence after chemomechanical preparation (S2) and intracanal dressing (S3) treatment.

Our findings suggest that the treatment procedures were less effective against non-pigmented Gram-positive organisms. The supposedly higher resistance of these kinds of Gram-positive bacteria (non-pigmented) may be related to various factors, for example, cell-wall structure, metabolic products secreted and resistance towards medicaments (Chavez et al. 2003).

A number of bacterial species were restricted to the posttreatment samples. Microorganisms may enter the canals during treatment because of leakage of rubber dam or temporary restorations, although we took extreme measures to prevent such occurrence. The fact that all control swabs were negative indicated contamination by leakage would be minimal. Apart from contamination, there are possible reasons why some bacterial species were only found in the posttreatment samples. For instance, different bacteria can localize at different depth of dentin, sampling of these bacteria is feasible only after preparation of the canal walls. Moreover, some of the bacterial species could not be identified in the preoperative samples if their cell number is below the detection limit by culture. If these microorganisms are resistant to treatment, changes in the intracanal environment may allow their multiplication to reach a detectable level after root canal instrumentation, irrigation and dressing (Chu et al. 2006).

This investigation detected *Enterococcus faecalis* in S1, confirming that it can be present in primary endodontic infections and, therefore, its persistence can lead to post-treatment disease. In fact, it is more associated with treatment failures.

In our study, the identification of the yeast *Candida albicans* from eleven canals, occurred more in S3 (five canals) samples, followed by S1 and S2, where only 2 cases were registered. A third of cases has treated with Ca(OH)₂ and were necrotic teeth. Fungi have occasionally been found in infected root canals that have not had any previous endodontic treatment, but they are more common in filled root canals in teeth that have become infected some time after treatment or in those that have not responded to endodontic treatment (Mohammadi & Abbot 2006). Overall, the occurrence of fungi reported in infected root canals varies between 1% and 17% (Waltimo et al. 2004). The effectiveness of intracanal dressings against *Candida albicans* was been widely studied (Waltimo et al. 1999) with conflicting results (Ercan et al. 2006). CHX is an effective antifungal agent especially against *Candida albicans* (Waltimo et al. 1999, Almyroudi et al. 2002, Basrani et al. 2003, Evans et al. 2003, Zamany et al. 2003, Menezes et al. 2004, Schafer & Bossmann 2005, Wang et al. 2007). *Candida albicans* seemed to be highly resistant to Ca(OH)₂ (Mohammadi & Abbot 2006).

CHX in a liquid form is a stronger disinfectant than CHX in gel form at various concentrations (Vianna et al. 2004) the reason for this performance may be related to dentine, dentine components (hidroxiapatite and collagen), killed microorganisms and inflammatory exudate in the root canal system may reduce or inhibit the antibacterial activity of CHX (Mohammadi & Abbot 2006).

Thus, rather than reliance on intracanal medicaments, thorough chemomechanical cleaning of the canal at the second visit is required to remove the residual canal flora (Chu et al. 2006).

7. Conclusions

The findings of this study indicate that endodontic treatment with different medicaments could markedly affect the diversity and quantity of cultivable microorganisms in infected canals, with some groups of microorganisms being more resistant to treatment than others. It was also noted that chemomechanical cleaning with the use of inter-appointment medicaments failed to render all the canals free from microorganisms.

The persistence of microorganisms inside the root canals may not lead to treatment failure. Further study about outcome, already running, is required.

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