Identification of Cultivable Microorganisms from Primary Teeth with Necrotic Pulps

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The objective of this study was to identify cultivable microorganisms from primary teeth with necrotic pulps. This experimental study included 21 patients of both sexes between 4 and 7 years of age with necrotic pulps in primary teeth. Twenty-one maxillary and mandibular molars containing at least 1 necrotic canal, an abscess or sinus tract, one or more radiolucent areas in the furcation or periapical region, teeth having at least two thirds of root length, and carious lesions directly exposed to the oral environment were included. After antisepsis of the oral cavity, anesthesia of the affected tooth, and isolation and disinfection of the operative field, 3 sterile absorbent paper points were sequentially placed for 30 seconds for the collection of samples. The samples were immediately processed in an anaerobic chamber, and all isolated microorganisms were identified. Anaerobic species (anaerobic facultative and moderate anaerobes) were isolated in all root canals; 68.4% of root canal samples studied showed a polymicrobial nature. Most of the isolate consisted of Bifidobacterium Spp2 and Streptococcus intermedius. Other less frequently encountered species were Actinomyces israelii, Bifidobacterium spp 1, Clostridium spp, and Candida albicans. Results indicate the existence of combinations of bacterial species in root canal infections of the primary dentition with necrotic pulps, anaerobic bacteria predominating.

Keywords: *anaerobic microorganisms, necrotic pulp, primary teeth.*
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INTRODUCTION

Microorganisms that invade the root canal system play an essential role in initiating and sustaining periapical disease, a common problem in the primary dentition. It may cause premature loss of teeth and its consequent sequelae, one of which is the failure in the functioning of the stomatognathic system. More than 700 bacterial species or phylotypes (of which over 50% have not yet been cultivated) have been detected in the oral cavity.[1] The bacteria present in infected root canals include a limited number of species compared with the total flora of the oral cavity.[2] It is now well-established that bacteria are the primary cause of the different forms of periradicular disease, and it has been demonstrated that endodontic infections are polymicrobial.[3,4] Some of them are asymptomatic but others are associated with serious symptoms.

Recently, through the development of anaerobic culture techniques and molecular studies, it has been possible to isolate anaerobic microorganisms associated with permanent teeth having necrotic pulps and periapical lesions. However, few studies have been concerned with identifying microorganisms from primary teeth with necrotic pulps.[5,6] Pulpal infections frequently are mixed, being composed of a restricted number of species per canal. Microorganisms usually form aggregates.[7] Sometimes interactions between different species can be beneficial to one or more microorganisms, while at other times they can be antagonistic. However, microbiologic studies conducted in different parts of the globe differ in the types of microorganism isolated.[8,9]

Numerous methods have been used for the identification of bacteria. The most widely used methods use morphological characteristics and determination of the metabolic

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properties of the unknown bacterium. These findings are compared with the properties of known bacteria in a database, and a presumptive identification is made.[10] The objective of this study was to identify cultivable microorganisms from primary teeth with necrotic pulps.

METHODS

Patients

Patients were recruited from the Clinic for Pediatric Dentistry Postgraduate Program, Facultad de Estomatologia, San Luis Potosí University, Mexico. The study was approved by the ethics committee of the university. The objective of the study was explained to either the parents or legal guardians, and written informed consent was obtained. This experimental study included 21 patients of both sexes between 4 and 7 years old. Inclusion criteria were as follows:

- Patient in good general health
- Mandibular and maxillary molars present
- Tooth containing at least one necrotic canal, abscess, or sinus tract
- Presence of radiolucent area(s) in furcation or periapical region
- At least two thirds of root remaining
- Carious lesion(s) without direct exposure to the oral environment
- Sufficient tooth structure to support a rubber dam
- Sufficient isolation and sterility control in operative field to demonstrate no bacterial growth

Patients who had received antibiotics up to 4 weeks prior to sampling were excluded.

Preclinical Laboratory Procedures

Prereduced thioglycolate tubes supplemented with hemin (5 mg L^{-1}) and menadione (1 mg L^{-1}) (Oxoid LTD, Basingstoke, Hampshire, England) were used as a transport medium owing to its capacity to maintain the vitality of sampled bacteria.[11]

Isolation and Operative Field Disinfection

The study procedure was performed by a pediatric dentist; periapical radiographs of the selected teeth were taken using the standard paralleling technique. After antisepsis of the oral cavity, local anesthesia was induced using an inferior alveolar nerve block for the mandibular primary molars and infiltration (palatal and buccal) for the maxillary primary molars. Each treated tooth was cleaned with pumice and isolated with a rubber dam. Provisit (Casa Idea, SA de CV, Mexico) was placed along the tooth–rubber dam interface to prevent leakage of saliva into the operative field. To disinfect the operative field, we followed the protocol previously described:[12,13] the tooth crown, surrounding rubber dam, and clamp were swabbed with 30% H_2O_2 (Fermont, Productos Quimicos Monterrey, Mexico) followed by 5.25% NaOCl for 1 minute each. The carious tissue was removed with a sterile round bur cooled with sterile saline solution. A sterile cotton pellet was placed on the floor of the chamber to prevent penetration of disinfectants into the canals, and, with another sterile bur, the root canal was accessed. The cavity and field were again disinfected as above. The NaOCl was inactivated with 10% sodium thiosulfate (Fermont) for 1 minute. Disinfection control samples were taken with sterile cotton pellets from the coronal surface of the tooth, rubber dam, and clamp, and immediately inoculated on blood agar plates (BBL Becton Dickinson, Mexico). The samples were then transferred to an aerobic incubator at 37ºC for 48 hours.

Collection of bacteriological samples

After estimating the canal length with the preoperative periapical radiograph, 3 sterile absorbent paper points of size compatible with root canal diameter (Viarden,Mexico), were sequentially placed for 30 seconds. If the canal was dry, then a small amount of sterile saline was used to wet the canal before the paper points were inserted. The retrieved paper points were immediately placed into the tube with thioglycolate. For the maxillary molars, samples were collected from the palatal root canal; for the mandibular molars, samples were collected from the distal root canal. After sample collection, the teeth were given conventional endodontic treatment.

Culture and identification of isolates

In the laboratory, samples were immediately processed inside an anaerobic chamber (85% N_2, 10% H_2, 5%CO_2) (Coy Laboratory Products, Grass Lake, Mich, USA). The entire 4-mL sample was vortexed at maximum speed (Kitlab, CM-101, India) for 1 minute. Each sample was inoculated on a 5% sheep-blood CDC anaerobic agar plate with vitamin K and hemin (BBL Becton Dickinson), incubated at 37°C, and observed every 24 hours until growth was present. Stains isolated were Gram-stained and classified by colony morphology, oxygen tolerance, and biochemical test. All microorganisms were characterized using identification kit API 20A (Analytical Profile Index, Biomeriéux, France). API Lab software (Biomeriéux) was used to ascertain strain identification on the basis of the numeric code generated.

RESULTS

All disinfection control samples yielded no growth, confirming effective disinfection of the operative field. All 21 samples contained cultivable microorganisms. The number of species per canal varied between 1 and 4 (mean, 2.3). Of the isolated species, 100% were moderately anaerobic and anaerobic facultative. The number of bacterial species isolated was 19 (Table 1).

There were 48 isolated bacteria recovered from 21 root canals. Most of the isolates consisted of *Bifidobacterium Spp2* (14 cases; 29.17%) and *Streptococcus intermedius* (6 cases; 12.5%). Other less frequently encountered species were 4 cases of *Actinomyces israelii* (8.33%), *Bifidobacterium spp 1*, *Clostridium spp,* and *Candida albicans*

Table 1. Cultivable microorganisms by sample

Sample	Microorganisms
1	*Lactobacillus acidofilus, Prevotella melaninogenica*
2	*Bifidobacterium spp2*
3	*Actinomyces naeslundii, Streptococcus intermedius, Bifidobacterium spp2*
4	*Bifidobacterium spp2*
5	*Streptococcus intermedius/constellatus*
6	*Bifidobacterium spp 2, Propionibacterium/propionicus*
7	*Bifidobacterium spp2*
8	*Bifidobacterium spp 2*
9	*Bifidobacterium spp 2*
10	*Actinomyces israelii, Bifidobacterium spp1*
11	*Clostridium beijerinckii/butyricum, Bifidobacterium spp2, Streptococcus intermedius, Candida albicans*
12	*Streptococcus intermedius, Clostridium ramosum, Bifidobacterium spp2*
13	*Bifidobacterium spp2, Propionibacterium acnes, Candida albicans*
14	*Streptococcus intermedius, Collinsella aerofaciens, Bifidobacterium spp2*
15	*Bifidobacterium spp2, Clostridium beijerinckii/butyricum*
16	*Bifidobacterium spp2*
17	*Streptococcus intermedius, Gemella morbillorum, Actinomyces naeslundii, Clostridium spp*
18	*Bifidobacterium spp2, Veillonella parvula, Actinomyces israelii*
19	*Actinomyces israelii, Bifidobacterium spp1, Clostridium tertium*
20	*Bacteroides ovatus/ thetaiotamicron, Candida albicans, Clostridium spp, Veillonella atipica*
21	*Bifidobacterium spp1, Clostridium spp, Actinomyces israelii*

Table 2. Frequency of bacterial species in 21 root canals

Species	Frequency	Percent
Bifidobacterium spp 2	14	29.17
Streptococcus intermedius	6	12.50
Actinomyces israelii	4	8.33
Bifidobacterium spp 1	3	6.25
Clostridium spp	3	6.25
Candida albicans	3	6.25
Clostridium beijerinckii	2	4.17
Actinomyces naeslundii	2	4.17
Lactobacillus acidofilus	1	2.08
Clostridium tertium	1	2.08
Clostridium ramosum	1	2.08
Prevotella melaninogenica	1	2.08
Propionibacterium acnes	1	2.08
Gemella morbilorum	1	2.08
Collinsella orofasciens	1	2.08
Propionibacterium propionicus	1	2.08
Bacteroides ovatus/thetaiotaomicron	1	2.08
Veillonella parvula	1	2.08
Veillonella atypica	1	2.08

Table 3. Main combinations of microorganisms

Combinations of species	Frequency
Bifidobacterium spp2-Streptococcus intermedius	4
Bifidobacterium spp1-Actinomyces israelii	3
Clostridium beijerinckii-Bifidobacterium spp2	2
Bifidobacterium spp2-Candida albicans	2

(6.25%), with 3 cases of each (Table 2).

A total of 13 (68.4%) combinations of bacterial species were isolated. Table 3 shows the main combinations that were found. *Bifidobacterium spp2-Streptococcus intermedius* combination was the most prevalent, with 4 cases.

DISCUSSION

Bacteria are the main etiologic agents of primary endodontic infections, which are caused by microorganisms colonizing the necrotic pulp tissue. Endodontic infections are mixed infections caused by normal inhabitants of the oral cavity, and multiple species have frequently been detected in association with disease.[14] Primary infections are mixed and predominated by anaerobic bacteria.

Maintaining the integrity of the primary dentition until normal exfoliation is vital for the development and maturation of the pediatric patient; for the growth of the facial-skeletal complex; and particularly for full development of the dental complex, its occlusion, esthetic qualities, and soft tissue support.[15] One of reasons for the premature loss of primary teeth is extraction caused by dental caries and root canal infection.

The ideal treatment in these cases is removal of the etiologic agent and endodontic therapy (pulpotomy or pulpectomy). These techniques are considered to be preventive since successfully treated teeth can be retained in a non-pathologic state until they exfoliate without endangering the permanent successor. The objective of root canal treatment is the complete elimination of the pathogenic microorganisms in primary teeth with an infected pulp. However, after endodontic treatment, some persistent microorganisms can cause treatment failures. Thus, it is essential to identify the microorganisms isolated from infected primary teeth so that the proper antimicrobial agents can be used locally to eliminate these pathogens.

In this study, anaerobic species (anaerobic facultative and moderate anaerobes) were isolated from all the root canals. This observation is in agreement with da Silva *et al* [5] who reported that, in 20 root canals of primary teeth with necrotic pulp and periapical lesions, anaerobic microorganisms were present in all cases.[5] However, they reported black-pigmented *bacilli* and *streptococci*. Also, our observations are in contrast to other reports in which the isolated bacteria were predominantly aerobic; they reported only 4 (20%) of samples with anaerobic bacteria (*Peptostreptococcus micros, Prevotella oralis*).[6] *Bifidobacterium Spp2* were found in 14 cases (29.17%) and *Streptococcus intermedius* in 6 cases (12.5%). Four canals were infected with *Actinomyces israelii* (8.33%) and 3 cases had *Bifidobacterium spp 1*, *Clostridium spp,* and *Candida albicans* (6.25%).

Other species isolated in low incidence included *Clostridium beijerinckii, Actinomyces naeslundii, Lacobacilus*

acidofilus, Clostridium tertium, Clostridium ramosum, Prevotella melaninogenica, Propionibacterium acnes, Gemella morbilorum, Collinsella orofasciens, Propionibacterium propionicus, Bacteroides ovatus thetaiotaomicron, Veillonella párvula, and *Veillonella atypica*. Some of these microorganisms have been isolated from root canals of permanent teeth with endodontic infection. Khemaleelakul *et al* reported the presence of anaerobic bacteria isolated from 17 acute endodontic abscesses and cellulites, such as *Streptococcus intermedius*, *Prevotella melaninogenica, Propionibacterium acnés, and Gemella morbilorum.*[16] *Bifidobacterium* was the most frequent microorganism isolated; bifidobacteria are normal ihabitants of the human digestive tract, and they are commonly used as probiotic bacteria.

Most of the species colonizing the oral cavity are transient colonizers.[17] Recently new species of *Bifidobacterium* have been reported, namely, *B adolescentis, B dentium, B longum,* and *B urinalis,* which were detected in the saliva of healthy humans. *B adolescentis* is associated with periodontal health status, specifically in young subjects.[18,19] *B dentium* is a species that inhabits the oral cavity and resides in deep periodontal pockets.[20] Another microorganism found with relative frequency was *Streptococcus intermedius*. This microorganism forms part of the normal flora of the oral cavity, gastrointestinal tract, and genitourinary tract, and it is often associated with purulent infections.[21] The streptococci group are early colonizers of dental plaque; they possess the ability to penetrate dentinal tubules and show potential adaptative responses to extreme environmental change.[22]

Interestingly, in this study, *Candida albicans* isolates were recovered from 3 (6.25%) cases. Adib *et al* reported *Candida spp.* in root canal samples of patients having persistent periapical disease and coronal leakage after endodontic treatment of permanent teeth.[23] *C albicans,* the fungal species most frequently isolated as an oral colonizer and pathogen, is a member of the indigenous microbial flora present in small numbers in the oral cavity of a large proportion of normal individuals wherein its growth is normally suppressed by others microorganisms.[24] This observation suggests investigating its possible clinical implications and to design novel therapeutic strategies in the field of filling materials used in endodontic treatment.

In addition, there have been no reports of the presence of certain bacteria that we isolated in the root canals of primary teeth. These findings might be due to the methods used in collecting of bacteria, and in culturing and identifying isolates. However, the possibility exists that the geographic region can be a factor in the composition of microbiota.[9] Some studies have suggested that genetic and environmental factors such as climate, eating habits, quality of drinking water, psychological stress, and access to and frequency of dental care can influence the composition of the oral microbiota.[25,26] Baumgartner *et al* and Siqueira *et al* have reported differences in the prevalence of bacterial species in root canal infections from distinct geographic locations.[8,9] These marked differences and our own findings suggest that species of bacteria could vary with geographic location.

The majority (68.4%) of root canal samples studied shown a polymicrobial nature. The *Bifidobacterium spp2-Streptococcus intermedius* combination was the most prevalent in 4 cases. Bacterial synergy is a crucial factor in bacterial adaptation to environmental stress. Bacterial interactions, the availability of nutrients, and the low oxygen tension in root canals with necrotic pulps can influence the growth and colonization of root canal bacteria.[27]

CONCLUSIONS

- These results indicate the existence of combinations of bacterial species, with a predominance of anaerobic bacteria, in root canal infections with necrotic pulps in the primary dentition.
- There is a possibility that the geographic region can be a factor in the type of microorganisms isolated in root canal infections of primary teeth.
- Knowledge of the microorganisms isolated from primary teeth with pulp infection has important clinical implications and suggests that further strategies in the field of endodontic filling materials be undertaken with the objective of selecting appropriate local antimicrobial agents to eliminate these pathogens.

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