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Correlation between bacterial type/bacterial quantity and bone loss detected by cone beam computed tomography (CBCT) in primary endodontic infections.

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Keywords: bone loss; cone beam; primary endodontic infection.

Abstract. Periapical lesions (PL) of endodontic origin are one of the most common pathological conditions that affect peri-radicular tissues. The main objective of this study was to evaluate the amount and species of microorganisms isolated from necrotic pulps, establish a correlation between these and the size of periapical lesions, and how the amount and species of microorganisms decreased with non-surgical root canal treatment. Twenty-seven patients with a clinical diagnosis of dental pulp necrosis and chronic periapical lesions were selected; a Cone Beam Computed Tomography (CBCT) and microbial samples of the root canal system were taken previous to a disinfection protocol, a post-instrumentation/disinfection protocol, and a post-medication placement. Samples were processed for colony-forming unit (CFU) counting, Gram staining technique, and bacterial identification by the API-20 Strep/API-20A system. The API system identified 21 species of microorganisms in the pre-instrumentation samples, 11 species in the post-instrumentation samples, and 11 in the post-medication samples. There was a correlation coefficient of 0.598% between the initial size of the lesion and the number of bacteria, with a coefficient of determination up to 35.7%, a correlation coefficient of 0.486% and a determination coefficient of 23.6% between the size of the periapical lesion and the number of CFUs. This study contributes to the knowledge of the amount and species of microorganisms isolated and identified from necrotic pulps, establishes a correlation between the amount and species of microorganisms and the size of the periapical lesions, and shows how the decrease of microorganisms contributes to the healing of PL, corroborating the importance of an adequate disinfection protocol.

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Correlación entre el tipo y cantidad de bacterias y la pérdida ósea detectada por Tomografía Computarizada de Haz Cónico (CBCT) en infecciones endodóncicas primarias.

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Palabras clave: pérdida ósea; haz cónico; infección endodóncica primaria.

Resumen. Las lesiones periapicales (LP) de origen endodóncico son la condición patológica más común que afectan los tejidos perirradiculares. El objetivo principal de este estudio es evaluar la cantidad y especie de bacterias aisladas de pulpas necróticas, correlacionar la cantidad y especies bacterianas con el tamaño de la lesión, y cómo disminuyen la cantidad y especies de microorganismos con el tratamiento de conductos. A 27 pacientes con diagnóstico de necrosis pulpar y lesión periapical crónica detectada con CBCT se les tomaron muestras microbianas del sistema de conductos antes y después del protocolo de desinfección y de la medicación intraconducto. Las muestras se procesaron para el recuento de unidades de formación de colonias (UFC), tinción de Gram e identificación mediante el sistema API-20 Strep/API-20A. Se identificaron 21 especies en las muestras pre-instrumentación, 11 en las muestras post-instrumentación y 11 en las muestras post-medicación; se observó un coeficiente de correlación del 0,598% entre el tamaño inicial de la lesión y la cantidad de bacterias, con un coeficiente de determinación hasta el 35,7%, un coeficiente de correlación del 0,486% y un coeficiente de determinación del 23,6% entre el tamaño de la lesión periapical y el número de UFCs. Este estudio contribuye al conocimiento sobre la cantidad y especies de microorganismos aislados e identificados a partir de pulpas necróticas, establece una correlación entre la cantidad y especies de microorganismos y el tamaño de las lesiones periapicales y exhibe cómo la disminución de microorganismos contribuye a la curación de LP, corroborando la importancia de un adecuado protocolo de desinfección.

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INTRODUCTION

Periapical lesions (PL) are one of the most common pathological conditions that affect peri-radicular tissues in the alveolar bone [1,2] and are mainly classified as radicular cysts or dental granulomas [1,3-5]. The microbial invasion and subsequent infection of the root canal system play a decisive role in the initiation, progression [1,4], and establishment of peri-radicular conditions[4] since bacteria and their by-products act as antigens that elicit a non-specific inflammatory response as well as specific immunological reactions in the peri-radicular tissues [2].

The bacterial species present in the apical region may have a significant role in the pathogenesis of apical periodontitis [6]. Gram-negative bacteria predominate in the root canal system of teeth with pulp necrosis and PL [6]. Some bacterial virulence factors include the structural components and products of bacterial metabolism [6]. It has been established that the levels of endotoxins in root canal infections are directly related to the severity of peri-radicular bone destruc-

tion [7]. Lipopolysaccharides (LPS) that form part of the bacterial cell wall and act like endotoxins are especially important in endodontic infections because of their biological effects, which lead to a complex interplay with host factors [6], like chemical mediators of inflammation, including the cytokines IL-1a, IL-1b, TNF-α and prostaglandins related to the pathogenesis of periapical lesions [5], resulting in clinical symptomatology, inflammatory reaction, and resorption of mineralized tissues[6]. Also, teichoic acid (TA) and lipoteichoic acid (LTA) are present in gram-positive bacteria and share their pathogenic properties with LPS, resulting in well-known injuries to the dental pulp and periapical tissues [6].

Most PL (>90%) can be classified as dental granulomas, radicular cysts, or abscesses [3], but the precise nature of such lesions can only be determined histologically; for this reason, the true prevalence of each pathological condition is unclear [5]. PL should be treated initially by a non-surgical approach [1]. The purpose of the non-surgical root canal treatment is to shape and clean the root canal system [1,4] to eliminate the necrotic tissue and infective bacteria and their antigens[1], and finally seal the root canal system three-dimensionally to prevent reinfection [4]. PL usually heals as a response to meticulous non-surgical endodontic treatment [4]. The primary root canal treatment yields predictable results with a survival rate of 95% after a 4-year follow-up [4]. To assess the healing potential of a PL, a period of 6 to 12 months after root canal treatment should be considered, while complete healing of the PL lesion might take up to four years. However, treatment failure is possible due to different microbial and non-microbial factors [4], leading to persistent intra- or extra-radicular infection, and a surgical procedure should be considered [1].

Nowadays, histopathological evaluation is the gold standard for diagnosing PL, but CBCT, MRI, and echography show promising results in differentiating granulomas and cysts [4]. In addition, CBCT offers relatively high-resolution and isotropic images. Potential applications in endodontics include diagnosis and evaluation of most aspects of endodontic treatment, such as determination of the configuration and length of the root canal, presence of accessory canals, and PL evaluation [8].

The main objective of this study was to evaluate the amount and species of microorganisms isolated from necrotic pulps, establish a correlation between these and the size of periapical lesions; and to determine how the amount and species of microorganisms decreased after the non-surgical root canal treatment.

METHODS

This study was evaluated and authorized by the Research Ethics Committee of the Faculty of Stomatology, UASLP, with the code CEI-FE-019-016.

Twenty-seven systemically healthy patients (18-60 years old) with a clinical diagnosis of dental pulp necrosis and chronic PL associate (primary endodontic infection) were selected for the study.

Microbial identification

Microbial samples of the root canal system were taken from each tooth previous to disinfection protocol, post-instrumentation/disinfection protocol, and post-medication placement.

Before each clinical procedure, the area of intervention was cleaned with a brush and Viarden® prophylactic paste (Viarden SA de CV, Mexico). Next, each patient was anesthetized with mepivacaine HCl 2% + epinephrine 1:100000. A rubber dam was placed and sealed with LC Block Out in the enamel rubber dam interface. The dental pulp chamber access was performed using a carbide bur #2, then the operative field was disinfected with hydrogen peroxide 30% (Fermont, Productos Químicos Monterrey SA de CV, Mexico) for 1 minute, sodium hypochlorite 2.25%

for 1 minute, and finally the solutions were inactivated with the application of sodium thiosulfate 10% for 1 minute (Fermont, Productos Químicos Monterrey, México).

The endodontic working length was established using an apex locator ID (SybronEndo, Kerr Corp. USA); then the pre-instrumentation bacterial sample of the root canal system was taken; after, the root canal was instrumented with Protaper Next rotary system (X1, X2, X3) (Dentsply Sirona, USA). The final irrigation protocol was used as follow: 2 mL of EDTA 17%, followed by 2 mL of NaOCl 2.25%, both solutions were activated with an E11 #25 ultrasonic tip and a Varios 370 ultrasound device (NSK, Shinagawa Tokyo, Japan); finally 3 mL of sterile saline solution were employed, a post-instrumentation sample was taken at this point. Finally, the intracanal medication was placed ($Ca(OH)_2$) and the dental pulp chamber was sealed with a temporary restoration (IRM, Dentsply). On a second session (7 days after session 1), each patient was anesthetized with Mepivacaine HCl 2% + epinephrine 1:100000. A rubber dam was used and LC Block Out was placed. The operative field was disinfected, and the temporary restoration was removed; then the intracanal medication was eliminated using ultrasonic tips; then the final irrigation protocol was performed as follows, 2 mL of EDTA 17%, followed by 2 mL of NaOCl 2.25%, both solutions were activated by an E11 #25 ultrasonic tip and a Varios 370 ultrasound device (NSK, Shinagawa Tokyo, Japan). Finally, 3 mL of sterile saline solution were used; the operative field was disinfected as previously described and the post-medication bacterial sample was taken.

All the bacterial samples were taken using a sterile Capillary Tip (0.035mm) (Ultradent®) connected to a 5 mL hypodermic syringe. The sample was placed in Eppendorf tubes with thioglycollate broth and incubated for 48 h in an anaerobic chamber (COY, laboratory products, Incubator Model 2000 Great Lake, USA).

Once all the bacterial samples were collected, the samples were incubated in an anaerobiosis chamber for 48 h, then samples were processed for CFU counting, Gram staining technique, and bacterial identification by API-20 Strep /API-20A.

CBCT evaluation

A CBCT of the involved teeth was taken before root canal treatment. The CBCT images were obtained by Kodak CS 9000 3D tomography equipment. The image size was established at 76 μm / 50 X 37 mm. Images were examined using Kodak CS3D (version 3.2.12) software.

Of the 27 periapical lesions diagnosed, ten were randomly selected and were measured using the PLM (Periapical Lesion Measurement Index) previous to the root canal treatment. Six months after non-surgical endodontic treatment, a second CBCT was taken to compare them. The bone volume destruction was determined using axial, sagittal, and coronal planes. The lesion's width, length, and depth were measured using the axial, sagittal, and coronal planes. The images were evaluated by a calibrated external observer using the Estrela's index[9]: 0 = Intact periapical bone structures, 1 = Radiolucency diameter 0.5-1 mm^3, 2 = Radiolucency diameter 1-2 mm^3, 3 = Radiolucency Diameter 2-4 mm^3, 4 = Radiolucency diameter 4-8 mm^3, Radiolucency diameter 8 mm^3, +E = Periapical cortical bone expansion, +D = Destruction of the periapical cortical bone.

RESULTS

Microbial identification (API-20 Strep/ API-20A)

The biochemical method API identified 21 species of microorganisms in the pre-instrumentation samples (Table 1), 11 species in the post-instrumentation samples (Table 2), and 11 in the post-medication samples (Table 3). The main microorganisms identified were *Actinomyces naeslundii* (16.66% pre-instrumentation/27.08% post-

Table 1
Bacteria species found in pre-instrumentation samples

		%
1	*Actinomyces naeslundii*	23.61
2	*Enterococcus faecalis*	16.66
3	*Aerococcus viridans*	8.33
4	*Streptococcus sanguis*	6.94
5	*Fusobacterium nucleatum*	6.94
6	*Clostridium spp*	5.55
7	*Streptococcus oralis*	4.16
8	*Aerococcus viridans 2*	2.77
9	*Porphyromona asaccharolytica*	2.77
10	*Bacteroides spp*	2.77
11	*Actinomyces israelii*	2.77
12	*Streptococcus intermedius*	2.77
13	*Aerococcus viridans*	2.77
14	*Propionibacterium propionicum*	1.38
15	*Streptococcus pneumoniae*	1.38
16	*Streptococcus mitis*	1.38
17	*Clostridium perfringens*	1.38
18	*Aerococcus otitis*	1.38
19	*Aerococcus urinae*	1.38
20	*Gardnerela vaginalis*	1.38
21	*Prevotella oralis*	1.38

Table 2
Species found in post-instrumentation samples

		%
1	*Enterococcus faecalis*	29.16
2	*Actinomyces naeslundii*	27.08
3	*Porphyromona asaccharolytica*	8.33
4	*PrevotelIa oralis*	8.33
5	*Fusobacterium nucleatum*	6.25
6	*Clostridium spp*	6.25
7	*Streptococcus oralis*	4.16
8	*Bacteroides spp*	6.24
9	*Aerococcus viridans*	2.08
10	*Streptococcus pneumoniae*	2.08

Table 3
Species found in post-medication samples

		%
1	*Actinomyces naeslundii*	40.00
2	*Enterococcus faecalis*	22.85
3	*Aerococcus viridans*	11.42
4	*Porphyromona asaccharolytica*	5.71
5	*Fusobacterium nucleatum*	2.85
6	*Bacteroides uniformis*	2.85
7	*Clostridium cadaveris*	2.85
8	*Clostridium perfringens*	2.85
9	*Bacteroides stercoris*	2.85
10	*Streptococcus sanguis*	2.85
11	*Prevotella*	2.85

instrumentation/40% post-medication), and *Enterococcus faecalis* (23.61% pre-instrumentation/29.16% post-instrumentation / 22.85% post-medication). Even when adequate instrumentation, proper disinfection, and use of intra-canal medication were carried out, species were detected on samples after non-surgical root canal treatment. This confirmed the decrease of bacteria after each therapeutic step procedure, but also showed that the complete disinfection of the root canal system is not possible.

For turbidity and CFU, descriptive statistic was performed (mean, mean error, standard deviation, minimum and maximum values) (Table 4) and compared between groups (Table 5). The normality of the variables was determined with the Shapiro-Wilk test. The student's T-test was used to compare groups. A Pearson's correlation coefficient was employed (95% confidence intervals).

According to the Gram stain, the bacteria most identified in the present study were Gram-positive.

CFU counting

According to the obtained results, based on turbidity, there is a correlation coefficient of 0.598% between the initial size of the lesion and the number of bacteria from

Table 4
Mean comparison of groups

	Sample	Mean	Mean Error	Standard Deviation	Minimum Value	Maximum Value
Turbidity	Pre-Shaping & Cleaning	4.826	0.330	1.713	2.500	7.500
	Post-Shaping & Cleaning	2.278	0.203	1.056	1.100	4.500
	Post-Intracanal Medication	0.937	0.063	0.328	0.500	1.600
CFU	Pre-Shaping & Cleaning	165.300	18.300	95.000	25.000	300.000
	Post-Shaping & Cleaning	48.440	4.720	24.500	6.000	100.000
	Post-Intracanal Medication	9.890	2.550	13.240	0.000	40.000

Table 5
Comparison of groups

	Pre-Shaping & Cleaning vs Post-Shaping & Cleaning	Pre-Shaping & Cleaning vs Post-Intracanal Medication	Post-Shaping & Cleaning vs Post-Intracanal Medication
Turbidity	0.001*	0.001*	0.001*
CFU	0.001*	0.001*	0.001*

*Statistical difference (P=≤0.001)

the pre-instrumentation sample, with a coefficient of determination up to 35.7%; a correlation coefficient of 0.486% and a determination coefficient of 23.6% between the size of the initial lesion and the number of total CFUs with statistically significant difference (* p = ≤0.001).

The quantification of CFU counting showed a bacterial decrease between the pre-instrumentation, post-instrumentation, and post-medication samples, with a statistically significant difference between all groups (Table 5), which means that each step procedure contributed to the control of the odontogenic infection.

CBCT evaluation

Ten random PL were evaluated by CBCT after six months of the endodontic treatment. According to the data obtained, there was a decrease in the volume size of the PL (Table 6).

DISCUSSION

The present study contributes to the knowledge about the amount and species of microorganisms isolated and identified from necrotic pulps, and determines a correlation between the initial size of a PL and the number of bacteria. It also corroborates that the root canal shaping, cleaning, and use of intracanal medication decreased the amount and species of bacteria identified, but do not eliminate them.

According to the obtained data, the bacteria most commonly identified from the root canal system were Gram-positive facultative anaerobes, corroborating previously reported literature [6]. It has been established that the microorganisms in the biofilm are exposed to very different environmental conditions from those in planktonic form, and many species can change their metabolism depending on the surrounding physiological and physicochemical conditions [10]. Periradicular dental biofilm is characterized by microorganisms adhered to the cementum, to the dentin, or both, in the apical portion of the root, surrounded by an external polysaccharide matrix (biofilm) that limits the access of defense molecules (antibodies and

Table 6
Comparison of periapical lesion before and after non-surgical root canal treatment

Sample	Initial Lesion Size	Initial PAI	Lesion Size 6 Months After Treatment	PAI 6 Months After Treatment	Reduction Percentage
1	8.20 mm^3	5	6.96 mm^3	4	15.12
2	13.80 mm^3	+D	7.90 mm^3	4	42.75
3	7.00 mm^3	4	4.59 mm^3	4	34.42
4	5.92 mm^3	3	3.19 mm^3	3	46.11
5	23.00 mm^3	+D	7.92 mm^3	4	65.56
6	8.96 mm^3	+E	4.34 mm^3	4	51.56
7	7.15 mm^3	4	4.60 mm^3	4	35.00
8	25.74 mm^3	+E	11.13 mm^3	+D	56.75
9	50.31 mm^3	+E	22.99 mm^3	+E	54.30
10	5.60 mm^3	4	3.04 mm^3	3	45.71

Estrela's Periapical Index (PAI): 0 = Intact periapical bone structures, 1 = Radiolucency diameter 0.5-1 mm^3, 2 = Radiolucency diameter 1-2 mm^3, 3 = Radiolucency Diameter 2-4 mm^3, 4 = Radiolucency diameter 4-8 mm^3, Radiolucency diameter 8 mm^3, +E = Periapical cortical bone expansion, +D = Destruction of the periapical cortical bone.

complement) and phagocytic cells (macrophages and neutrophils) [6]. The microorganisms forming biofilms are more resistant to antimicrobials (up to 1000 times less susceptibility to specific antimicrobials) and the host immune defenses than their planktonic counterparts [10].

The purpose of root canal treatment is to debride and disinfect the root canal system and to eradicate intracanal bacteria or at least reduce them to a level below that necessary to heal and prevent periapical diseases or allow their resolution [11]. However, in some cases when the apical seal fails (apical filtration), the pathology persists [12], due to residual microorganisms [4], or extra-radicular microorganisms [6], both with access to periradicular tissues maintaining the pathology[4]. In primarily infected root canals, microorganisms were able to access and colonize the pulpal tissue and impair its function [6]. The most common pathologic factors in the alveolar bone derived from necrotic dental pulp are PL [13]. Their microbial profile consists of 10-30 species per canal [6]. According to the data obtained in our study, at least 21 different species of microorganisms were identified previously to the root canal disinfection. The species number identified post-disinfection and post-medication decreased to 11 species.

It has been reported that microorganisms like *Fusobacterium, Porphyromonas, Prevotella, Parvimonas, Tannerella, Treponema, Dialister, Filifactor, Actinomyces, Olsenella*, and *Pseudoramibacter* predominated in the root canal system; also, some facultative or microaerophilic streptococci are commonly found in primary infections [6]. According to our data, the main microorganisms identified were *Actinomyces naeslundii, Enterococcus faecalis, Aerococcus viridans, Streptococcus sanguis,* and *Fusobacterium nucleatum*, corroborating previous reported literature. Cardoso *et al*. [7] revealed a positive correlation between root canal volume, determined by CBCT analysis, and CFU count found in primary endodontic infections with apical periodontitis [7]. It also showed that the presence of selected bacteria species, such as *L. buccalis, P. intermedia, C. gracilis, C. gingivalis,* and *C. sputigena*, as well as

their interaction in the form of complexes, was positively correlated with the presence of clinical features. Cardoso *et al.* also revealed that larger root canals hold higher levels of culturable bacteria. Thus, the interaction of different virulent bacteria species in complexes plays an important role in the development of clinical features[7]. This data is corroborated by the results reported in this study, in which there is a relation between the number of bacteria and the presence of a PL. In a study performed in Taiwan by Li-Wan Lee [14], it was found that the main species of bacteria identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry, were *Porphyromonas endodontalis, Bacteroides fragilis, Dialister invisus, Fusobacterium nucleatum* and *Treponema denticola* [14], the differences of main species reported could be due to the difference of population evaluated and the laboratory techniques to isolate, culture and identify the microorganisms. One of the main reported specie is *E. faecalis*, a facultative gram-positive bacterium, capable of surviving in an environment with scarce availability of nutrients and minimal commensality with other bacteria. It presents different virulence and resistance mechanisms, which hinder its eradication from root canals [6].

Literature has reported that at a 6-month follow-up after the root canal treatment, only half of the cases exhibit signs of healing and that after a 12-month interval, 88% of these lesions exhibit signs of recovery. In contrast, complete healing of the peri-apical lesion might take up to four years [4]. Our study corroborates these findings since the samples evaluated by CBCT 6 months after the root canal treatment showed a size reduction of the PL. The control and resolution of the associated infection and healing of PL depend on different factors, including the amount and species of bacteria related to the infection process and the capability of the immune system to control the remaining bacteria. Interactions of bacteria species and their grouping into complexes make endodontic infections even far more complex for the immune system response, which can lead to different clinical symptoms [7].

It has been reported that lesions ≤ 10 mm had an 80% of success rate while the larger ones showed a success rate of 53% [12], then the largest periapical lesions are associated with the worst prognosis [12]. In our study, it was possible to prove a directly proportional relationship between the lesion size and the amount and number of bacterial species. Also, it has been established that the pathologic nature of the PL plays an essential role in the clinical evolution of the periapical disease; a true periapical cyst is less likely to heal after non-surgical root canal treatment and might require peri-radicular surgery [4].

A definitive diagnosis of peri-radicular cyst is reached only through histopathologic evaluation [4,13] by serial cross-sectioning of the lesion specimen [4]. But nowadays, the CBCT represents a non-invasive method for differentiating periapical cysts and granulomas[4]. Also, represents an ideal method to evaluate the healing of a PL after root canal treatment or surgical endodontic treatment, consistent with the data reported in this study. Nevertheless, according to the American Association of Endodontists (AAE), CBCT should only be used when the required imaging question cannot be answered adequately by lower-dose conventional radiography or alternate imaging modalities [4].

The comprehension and understanding of the microbial characteristics in the root canal system play an essential role in the treatment and resolution of periapical diseases. This study determined that the amount and species of microorganisms isolated from necrotic pulps, established a correlation between the amount/species of microorganisms and the size of periapical lesions, and showed that the decrease of microorganisms through the non-surgical root canal treatment contributes to the healing of PL, corroborating the importance of an adequate disinfection protocol. It also established that the CBCT

could be used as an objective method to evaluate the evolution of a PL after root canal treatment. However, further studies are needed to confirm the data reported.

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Conflict of Interest

The authors declare not to present any conflict of interest or competence with the research work carried out.

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