Revista Biomédica

http://revistabiomedica.mx

Artículo original

Presence of red complex bacteria and *Aggregatibacter actinomycetemcomitans* in necrotic primary teeth with periapical abscess in children of Sinaloa, México

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RESUMEN

Presencia de bacterias del complejo rojo y *Aggregatibacter actinomycetemcomitans* en dientes primarios necróticos con absceso periapical en niños de Sinaloa, México.

Introducción. La necrosis pulpar es uno de los principales motivos por los cuales se realizan tratamientos en dentición infantil. El complejo rojo y *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* se han asociado con patología pulpares.

Objetivo. Evaluar la presencia del complejo rojo y *A. actinomycetemcomitans* en placa bacteriana, canal radicular y abscesos periapical en infantes 3 a 9 años de edad con diagnóstico de necrosis pulpar.

Materiales y métodos. Las muestras de los tres sitios fueron preservadas y los microorganismos fueron identificados por PCR.

Resultados. Veinte seis niños (5.5 ± 1.7 años) fueron evaluados, 92.3 % fueron positivos para una bacteria en por lo menos uno de los lugares evaluados. El microrganismo más prevalente fue *T. forsythia* (76.9 %) y el menor *T. denticola* (11.5 %). Sin embargo, *A. actinomycetemcomitans* no fue encontrada en ninguna de las muestras. La placa bacteriana fue el lugar con mayor frecuencia de bacterias (80.7 %, $p < 0.001$). Un diagrama simétrico fue realizado dando como resultado que *T. forsythia* se relacionó con el sitio de placa bacteriana, mientras *T. denticola* con fistula y *P. gingivalis* con canal radicular.

Conclusiones. Las bacterias del complejo rojo (*P. gingivalis, T. denticola y T. forsythia*) fueron encontradas en una alta frecuencia en los tres sitios analizados. *A. actinomycetemcomitans* no fue encontrada en ninguna muestra. Con estos resultados se observa que dependiendo de la zona anatómica se modifica la presencia de estos

Historial del artículo
Recibido: 28 oct 2019
Aceptado: 14 mar 2020
Disponible en línea: 1 may 2020

Palabras clave
Complejo rojo, dientes temporales, absceso periapical.

Keywords
Red complex, primary teeth, periapical abscess.

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microorganismos, conocer esto es esencial para elegir apropiadas técnicas de irrigación capaces de inhibir la proliferación bacteriana y así lograr el éxito clínico.

ABSTRACT

Introduction. Pulp necrosis is a common reason for root canal treatment in primary teeth. Pulpal Pathologies has been associated with the red complex and *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans.*

Objective. The study evaluated the prevalence of the red complex and *A. actinomycetemcomitans* in dental plaque, root canal, and periapical abscesses in children from 3-to-9 years old with necrotic pulp.

Materials and methods. Samples of the three sites were preserved and microorganisms were identified by PCR.

Results. Twenty-six children (5.5 ± 1.7 years old) were evaluated, 92.3 % were positive for bacteria at least in one place. *T. forsythia* was the most prevalent (76.9 %) and *T. denticola* the least (11.5 %). However, *A. actinomycetemcomitans* was not found in any sample. The bacterial plaque was the most prevalent place of bacteria (80.7 %, $p < 0.001$). Interestingly, *T. forsythia* was related with bacterial plaque site, while *T. denticola* with fistula and *P. gingivalis* with root canal.

Conclusion. The bacteria of the red complex (*P. gingivalis, T. denticola and T. forsythia*) were found in high frequencies in the three evaluated sites. *A. actinomycetemcomitans* was not found. With this resutls, it can be seen that, depending on the anatomical area, the presence of this microorganism will be modified. This kwnoledge is essential to choose irrigation techniques capable to inhibite bacterial proliferation and achive clinical success.

INTRODUCTION

The development of pulp necrosis in primary teeth is due to the entry and multiplication of oral microorganisms in the pulp tissue (1). The bacteria arrive at the pulp cavity, mainly due to their exposure through active carious lesions (2). When pulp necrosis is established, infection quickly spreads towards the periradicular tissue (3).

Periodontal bacteria are classified according to their appearance and association in the development of the disease into six groups (4). The red complex group composed by *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia,* has been considered the most pathogenic microbial complex in periodontitis (5) and associated in pulpal pathologies (6-9). Besides, *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) is also considered a classic periodontal pathogen (5).

Several studies have focused on evaluating the microbiota associated with infections in adults (10-12). Gomes G.B. assessed the presence of endodontic pathogens, including the red complex, in necrotic primary teeth, detecting *P. gingivalis*, *T. forsythia* and *P. gingivalis* in pulp chamber and root canal (6). Pourhajibagher M. found high frequencies of *P. gingivalis* (14.1 %) and *A. actinomycetemcomitans* (9.2 %) in endodontic infections (13). Despite that these bacteria are related to the etiology of periapical abscess, several studies analyze their prevalence in necrotic primary teeth (2, 12, 14-21). However, the treatment of these teeth is performed in dental practice routinely. Red complex pathogens have been reported with a prevalence range of 16 to 49 % of *P. gingivalis* (14, 18, 19, 21), 16 to 48.3 % of *T. denticola* and 1.6 to 56.2 % of *T. forsythia* (2, 14, 19) in primary teeth. Therefore, knowing the microbiota of the necrotic pulp allow the proper use of antimicrobial agents (14, 15) for the control of the infection since the vast spaces of the medular bone could allow their dissemination (15). Furthermore, since the infection can reach the germ of the permanent tooth, it is of the utmost importance to preserve it healthy (2, 22).

This study aimed to evaluate the prevalence of periodontal bacteria of the red complex and *A. actinomycetemcomitans a* in bacterial plaque, root canal, and periapical abscess of primary teeth.

 https://doi.org/10.32776/revbiomed.v31i2.779

MATERIALS AND METHODS

Patients.

63 infants aged between 3 and 9 years were evaluated, from them, 26 with dental infection presented the inclusion criteria: pulp necrosis and periapical abscess with radiographically detected periradicular/interradicular bone resorption and less than 2/3 degree of physiological root resorption in primary teeth, and not be under antibiotic treatment for at least 3 months. Twenty-five primary teeth were molars, and one was single-rooted teeth. Samples from multi-rooted teeth were taken from the largest root canal, always associated with the periapical lesion. The children from the Department of the Pediatric Dentistry, Universidad Autónoma de Sinaloa (UAS), and the Hospital Pediátrico de Sinaloa "Dr. Rigoberto Aguilar Pico" were treated and were excluded those with any systemic disease. Informed consent was following the Declaration of Helsinki in 2008 without any economic retributions. The research committee of the Department of Dentistry from the Universidad Autónoma de Sinaloa approved the study. The same pediatric dentist was trained to evaluate the tooth, clinical parameters and sampling.

Bacterial plaque sample.

The bacterial plaque sample was collected from the affected tooth after cleaning the crown with sterile gauze, using a sterile curette (Gracey curet, Hu-Friedy, Illinois, USA) at 4 sites around the tooth (vestibular, mesial, lingual and distal). The samples were placed in 1 ml of sterile 1X PBS and stored at -80 °C for later processing according to the method published by Soto-Barreras, Olvera-Rubio (23).

Sample of necrotic pulp and periapical abscess.

The periapical abscess samples were collected after the application of local anesthesia and antisepsis of the oral cavity with sterile gauze. The purulent content was taken with an insulin syringe of 100 units 1 cc/mL sterile. The samples were placed in 1 ml of sterile 1X PBS and stored at -80 °C until processing.

On the other hand, for the root canal samples, the affected tooth was isolated with a rubber dam and brushed with 3 % hydrogen peroxide and decontaminated with 1 % sodium hypochlorite. The decayed tissue was removed using sterile carbide ball burs # 6, and complete access preparation was carried out with a new high-speed sterile carbide bur # 6 with sterile water irrigation. A sterile Hedstroem file # 25 of 21 mm (Readysteel Senseus, Dentsply Maillefer, Ballaigues, Switzerland) was introduced into the canal up to approximately 1 mm short of the tooth apex in cases with intact roots, or to the limit of physiologic root resorption. Rotary movements were performed for 60 s and then transferred to the tube with 1 ml of sterile 1X PBS. The samples were stored at - 80 °C until use. All the teeth involved in the sampling process were subjected to a pulpectomy, following the American Pediatric Dentistry Association protocols.

Extraction.

DNA was purified using Puregene DNA isolation kit (Gentra Systems, Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer instructions. Briefly, 500 μL of sample were centrifuged for 10 min at 16,000 rpm (Microcentrifuge 5417R, Eppendorf, Hamburg, Germany), the supernatant was discarded, and the pellet resuspended in 200 μL of lysis buffer (1.0 % Triton X-100, 20 mM Tris-HCl, 2 mM EDTA pH 8.0) and placed in a water bath at 85 °C for 10 min. Then, 100 μL of mutanolysin (200 U/ml, Sigma-Aldrich, Missouri, USA) was added and incubated at 50 °C for 1 h. 60 μL of K protein (Promega, Wisconsin, USA) was added and incubated at 80 °C for 10 min. 60 μL of 0.75 M ammonium acetate was added and vortexed for 20 s. Afterward it was centrifuged at 13,000 rpm for 10 min. and the supernatant placed into tubes and 500 μL of phenol:chloroform:isoamilic alcohol solution (25:24:1, Invitrogen, California, USA) was added and homogenized by soft inversion. The samples were centrifuged and the upper phase transferred to a new tube. 400 μL of isopropanol was added, and placed in freezing for 10 min, following

by centrifugation at 13,000 rpm discarding the supernatant. 500 *µL* of 70 % ethanol was added and centrifuged at 13,000 rpm, and the supernatant discarded. Finally, DNA was resuspended in 50 µL of TE buffer, and the concentration calculated using a NanoDrop One (Thermo Fisher Scientific, Massachusetts, USA).

Detection of bacterial by PCR.

The specific primers (24-26) for the evaluated bacteria are in Table 1. As positive controls, the DNA of P. gingivalis (ATCC 33277), T. forsythia (ATCC 43037), T. denticola (ATCC 35405), and A. actinomycetemcomitans (ATCC 29523) strains were used. 25 *µl* of master mix (1X reaction buffer, 2.0 mM MgCl2, 0.2 *µM* dNTP's, 0.1 *µM* of each primer, 1U of Taq DNA polymerase and 50 ng of DNA) was subjected to amplification (30 cycles of 1 min at 95 °C, 1 min at 56 °C and 1 min at 72 *°C) in a T100 thermal cycler (Bio-Rad, California, USA). The amplicons* were corroborated in 1.5 % agarose gels (27).

Table 1. Sequences of primers used to identify four bacterial species in the different anatomical sites evaluated.

Bacteria	Primers (5'-3')	M	Ref
P. gingivalis	ATGTAGATGACTGATGGTGAAAACC ACCATCCCCACCTTCCTC	197	24
T. forsythia	GCGTATGTAACCTGCCCGCATGC TTCAGTGTCAGTTATACCT	641	25
T. denticola	TAATACCGAATGTGCTCATTTACAT TCAAAGAAGCATTCCCTCTTCTTC	316	25
A. actino-mycetem-comitans	GCCGACACCAAAGACAAAGTCT GCCCATAACCAAGCCACATAC	262	26

M: Molecular Size (bp)**, Ref:** References

Statistical analysis

The descriptive statistical analysis was carried out by frequency and percentage in qualitative variables. A Chi-square test was used. to determine differences among groups. To describe the relationships between bacteria and the sites analyzed (bacterial plaque, root canal, and periapical abscess), a dimension analysis was made through simple correspondence analysis for multidimensional visualization among the variables. Statistical significance was established with an alpha of 0.05, using the statistical package IBM SPSS Statistics 23.0 (IBM, Nueva York, USA).

RESULTS

The average age of infants was 5.5 ± 1.7 years, of which 38.4 % were male and 61.5 % female. The most common primary teeth with abscess were the first molar with 61.5 % (34.6% in maxillary molars and 26.9 % in mandibular molars), the second molar with 34.6 % (3.8 % in maxillary molars and 30.8 % in mandibular molars), and the incisors with 3.8 %. 46.15% reported some pain related to the pathological process.

The presence of the red complex bacteria in at least one of the three sites analyzed (bacterial plaque, root canal, and abscess) was found in 92.3 % of the infants Furthermore, 80.7 % presented at least one bacteria in bacterial plaque, 46.3 % in the root canal, and 30.7 % in abscess ($p < 0.001$). In samples of bacterial plaque, *T. forsythia* was the most prevalent, with 76.9 % ($p < 0.001$) (Table 2). *P. gingivalis* was present in 30.8 %, and *T. denticola* in 11.5 % of samples (Table 3).

In the area of the abscess, *T. denticola* showed higher frequency of 23.1%, while *P. gingivalis* of 11.54 %. The infants that presented the higher frequency was male from 7 to 9 years, with 23.1 % (Table 3). Moreover, *P. gingivalis* was also found in 23.1% of samples of the root canal. The most affected children were female, with an age range of 3 to 6 years (Table 3). On the other hand, *A. actinomycetemcomitans* was not found in any of the samples evaluated in this study. Figure 1 shows correspondence analysis plot of the type of bacteria and the location of the analyzed samples. Correspondence analysis allows the multivariate treatment of data by simultaneously considering multiple categorical variables.

 https://doi.org/10.32776/revbiomed.v31i2.779

Table 2. Prevalence of bacteria according to the sample collection site.

Variable	Frequency	Percentage	*p*
Bacterial Plaque			
P. gingivalis	8	30.8	
T. forsythia	20	76.9	<0.001
T. denticola	3	11.5	
Dental Abscesses			
P. gingivalis	3	11.5	
T. forsythia	4	15.4	0.524
T. denticola	6	23.1	
Root Canal			
P. gingivalis	6	23.1	
T. forsythia	3	11.5	0.412
T. denticola	3	11.5	

Table 3. Presence of bacteria according to age, sex and site of collection of the sample in the study population.

Bacteria	**3 to 6 years**		**7 to 9 years**		
	M	F	M	F	n (%)
Bacterial plaque					
P. gingivalis	0	5	2	1	**8 (30.8)**
T. forsythia	4	11	4	1	**20 (76.9)**
T. denticola	0	1	2	0	**3 (11.5)**
n (%)	4 (15.4)	**17 (65.4)**	**8 (30.8)**	**2 (7.7)**	
Fistula					
P. gingivalis	1	0	2	0	**3 (11.5)**
T. forsythia	0	2	2	0	**4 (15.4)**
T. denticola	1	3	2	0	**6 (23.1)**
n (%)	**2 (7.7)**	**5 (19.2)**	**6 (23.1)**	**0 (0)**	
Root canal					
P. gingivalis	0	4	2	0	**6 (23.1)**
T. forsythia	0	2	1	0	**3 (11.5)**
T. denticola	0	1	1	1	**3 (11.5)**
n (%)	**0 (0)**	**7 (27.0)**	**4 (15.4)**	**1 (3.8)**	

M= Male F=Female

Interestingly, some clear groupings were determined, *T. denticola* was grouped with periapical abscess, *T. forsythia* was the closest to bacterial plaque, and *P. gingivalis* was in the same quadrant of the map as a root canal. It is quite clear that different collection sites were related to different bacteria. This analysis can reveal relationships that would not be detected in a series of comparisons of variables in pairs.

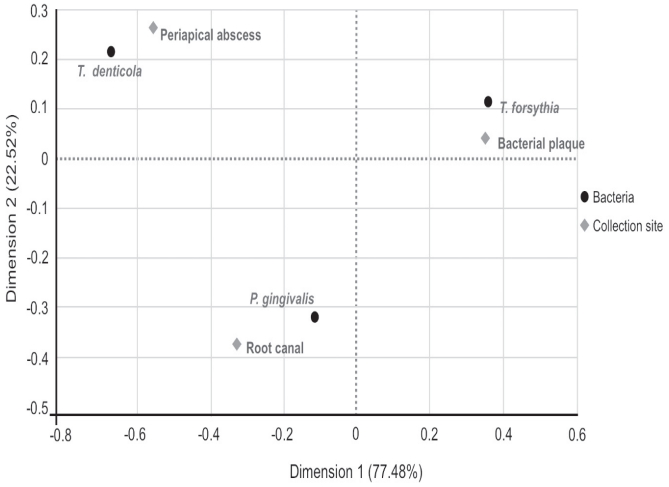

Figure 1. Simple correspondence analysis of presence of the red complex bacteria by collection site

DISCUSSION

Infections in the root canal involve a mixture of microorganisms with a predominance of anaerobic bacteria (6). Recently, some studies have focused on the relationship between bacteria associated with periodontal diseases and their connection to systemic diseases as well as to pulpal pathologies. Several reports have found a high frequency of these species in saliva and ~~in~~ the oral cavity biofilm (28 - 30). The present study assessed the prevalence of periodontal pathogens of the red complex and *A. actinomycetemcomitans* in necrotic primary teeth in three infection sites: bacterial plaque, necrotic root canal, and periapical abscess. Previous studies have suggested that red complex associated infections show a higher risk of complications such as periapical lesions, abscess, and cellulitis, bone resorption, acute clinical symptoms, pain, and inflammation (31).

Anaerobic bacteria were evaluated and detected as described by other authors (15, 17). These reported that individuals with necrotic pulp in root canals of primary teeth and periradicular lesions showed

a polymicrobial infection with a predominance of anaerobic microorganisms, similar to the microbiota of permanent teeth. Some studies have reported about 60 % presence of anaerobic bacteria (32) in the root canal of primary teeth. However, our study showed the presence of these bacteria in 46.3% of the samples. This could be due to the difference in the methodology and the amount of evaluated bacterial species.

Gomes y col. (6) reported the presence of *P. gingivalis* in 100 % of the root canal samples analyzed. Conversely, other authors have reported that the presence of *P. gingivalis* in endodontic infections of primary teeth is approximate of 10% (33) and 29.6% (34), similar to our study, with 21.79 % of prevalence in the three evaluated sites. *P. gingivalis* was present in 30.77 % of the samples of bacterial plaque, 23.08 % in root canal, and 11.54 % in abscess.

In the case of *T. denticola,* this bacterium has been reported with a presence from 2.3 to 3.4 % in abscess of necrotic primary teeth (18, 32). We found it in 23.08 % of the samples, and we believe that this difference is due to the methodology used to take the sample. In the previously published, authors used paper tips, in our case we puncture the abscess getting a larger amount of sample. Some similar was observed with *T. forsythia*, which has been reported with a presence of 2.3 % (16, 18) in abscesses; we found a frequency of 15.38 % of this bacteria.

According to our results, the anaerobic bacteria evaluated are frequently present in the microbiota of pulpal infections of primary teeth. Interestingly, *T. forsythia* was more prevalent with bacterial plaque site, while *T. denticola* with abscess and *P. gingivalis* with root canal. We consider that this may be due to the presence of extensive and deep caries and the poor level of oral hygiene (35).

In the case of *A. actinomycetemcomitans,* none of the samples evaluated presented this bacteria. In bacterial plaque, we coincide with Conrads y col. (36) that no detected *A. actinomycetemcomitans* in a kindergarden (3 to 5 years) and primary school (9 to 10 years) children using PCR; however, has been detected in children from 2 to 13 years of old using checkerboard DNA–DNA hybridization technique (37) and PCR (38). We consider that this discrepancy could be related with the differences between populations. In the root canal has been reported that this microorganism is not able to survive (18); this statement is supported by our results.

The importance of knowing the microbiota of necrotic primary teeth is essential for pediatric dentists to choose the appropriate irrigation techniques and treatments that are capable of inhibiting bacterial proliferation and achieve clinical success. We highlight the importance of properly reducing the microbial population with irrigants as sodium hypochlorite, always avoiding extrusion beyond the apex.

More clinical studies are needed to evaluate if the failures in the therapy of necrotic primary teeth are due to the permanence of the bacteria of the red complex, as well as the implementation of modern and advanced molecular assays that provide a clear view of the microbiota in primary dentition infections, this with the purpose to improve the treatments. The main limitation of this study was the number of patients due to the strict inclusion criteria.

CONCLUSIONS

The bacteria of the red complex (*P. gingivalis, T. denticola and T. forsythia*) were found in high frequencies in the three evaluated sites. *A. actinomycetemcomitans* was not found. *T. forsythia* was more prevalent in bacterial plaque site, while *T. denticola* in abscess and *P. gingivalis* in root canal. Most studies published so far have focused on evaluating the root canal. We can observe how the presence of microorganisms changes depending on the anatomical site; even so, it is necessary to confirm these results with a larger sample size.

ACKNOWLEDGMENT

To the Pediatric Hospital of Sinaloa "Dr. Rigoberto Aguilar Pico ", for allowing us to work with their patients and their facilities during the sample collection process.

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