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Detection of endodontic pathogens in root canals of deciduous teeth by molecular techniques

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Aim: This study aimed to assess the efficacy of two molecular methods, namely PCR assay and checkerboard DNA-DNA hybridization, for the detection of specific endodontic pathogens in the root canals of deciduous teeth with primary endodontic infection. It also aimed to investigate the most prevalent species detected regardless of the method used. **Methods:** Samples were taken from 44 cases of deciduous teeth with primary endodontic infection. DNA was extracted and the presence of microorganisms was checked by using PCR assay and checkerboard DNA-DNA hybridization assay. Fisher´s exact test was used to observe the possible difference in the prevalence of each pathogen concerning the molecular methods. Sensitivity, specificity, and accuracy tests were performed between both methods. The statistical significance level was 5%. **Results:** In our study, PCR and checkerboard DNA-DNA hybridization had specificity higher than sensitivity, demonstrating that these assays can identify true-negative samples. Both methods showed a similar capacity to detect specific endodontic pathogens in root canals of deciduous teeth with primary endodontic infection. PCR and checkerboard showed no statistically significant differences for most species detected, except four species: *P. micra* and *P. nigrescens*, which were more prevalent by PCR, and *F. alocis* and *P. intermedia*, which were more prevalent by checkerboard DNA-DNA hybridization. **Conclusions:** In conclusion, both methods are efficient in detecting endodontic pathogens and one should complement the other. Taking together the results from both methods, *A. naeslundii* and *F. nucleatum* were the most prevalent species.

Keywords: Infections. Polymerase chain reaction. DNA probes. Tooth, deciduous. Endodontics.

Introduction

Microorganisms are the main etiological agent of endodontic infections[1]. Bacterial species and their levels in the root canals may influence the success of the endodontic treatment[2,3]. After pulp necrosis, microorganisms may migrate from the root canal to the periapical area, inducing necrosis of bone and soft tissue and accumulation of purulent exudates[4]. In primary teeth, infection in the periapical area can damage the underlying permanent tooth germ and affect its development, in addition to being a source of serious orofacial infection[5]. Therefore, knowing the microorganisms present in root canal infections is important to establish an effective antimicrobial treatment for the prevention of the spread of infection through the periradicular spaces.

Molecular biology has been performed to investigate the microbiota in deciduous teeth with pulp necrosis[6-9], in which the existing techniques improved extraordinarily the recognition of microorganisms associated with endodontic diseases[10]. In fact, the use of these molecular methods has allowed a better determination of the microorganisms present in endodontic infections by giving recognizable proof of certain microorganisms not detected by culture methodology[11].

Polymerase chain reaction (PCR) was the primary strategy used in molecular studies to amplify nucleic acid sequences[10]. PCR has been widely used for the detection of microbial pathogens as it enables to isolate any gene from any organism[12]. The technique comprises repetitive cycles of DNA denaturation, annealing, and extension by using DNA polymerase[13]. The result of each PCR cycle is a newly synthesized double DNA[13]. PCR is one of the preferred methods in which nucleic acid amplification is used for its widely approved standard sensitivity, including accessibility of reagents and materials[14]. However, it requires precise handling and can be affected by sample contamination.

The checkerboard DNA–DNA hybridization has been reported to be a fast and sensitive molecular assay[15,16]. It overcomes numerous disadvantages of the culture method, such as loss of viable microorganisms amid transport, difficulty in cultivating certain species, and difficulty in identifying some species with only a few phenotypic characteristics[16]. One more positive point is that the whole sample might be used without dilution or amplification, thus resolving issues that may occur with PCR. At last, the procedure gives semi-quantitative information that might be significant in the treatment of biofilm-related diseases, where species levels may be reduced by the treatment, but not completely eradicated[16].

This study aimed to assess the efficacy of two molecular methods, PCR assay and checkerboard DNA-DNA hybridization, for the detection of specific endodontic pathogens in the root canals of deciduous teeth with primary endodontic infection. Additionally, it aimed to investigate the most prevalent species detected, regardless of the method used. While previous studies have compared these two methods[17], our study expands on this by examining a greater number of species, including 14 bacterial species, and by showing the levels of bacteria detected through the semi-quantitative method, thus providing new insights into the microbiota of end-

odontic infections in primary teeth. By comparing PCR and checkerboard DNA-DNA hybridization, this study aims to provide a thorough evaluation of these techniques, offering valuable information for improving the diagnosis and treatment of endodontic infections in primary teeth.

Materials and Methods

Patient Selection

The patients were enrolled and allocated and enrolled for this study according to the STROBE guidelines, as described in Figure 1.

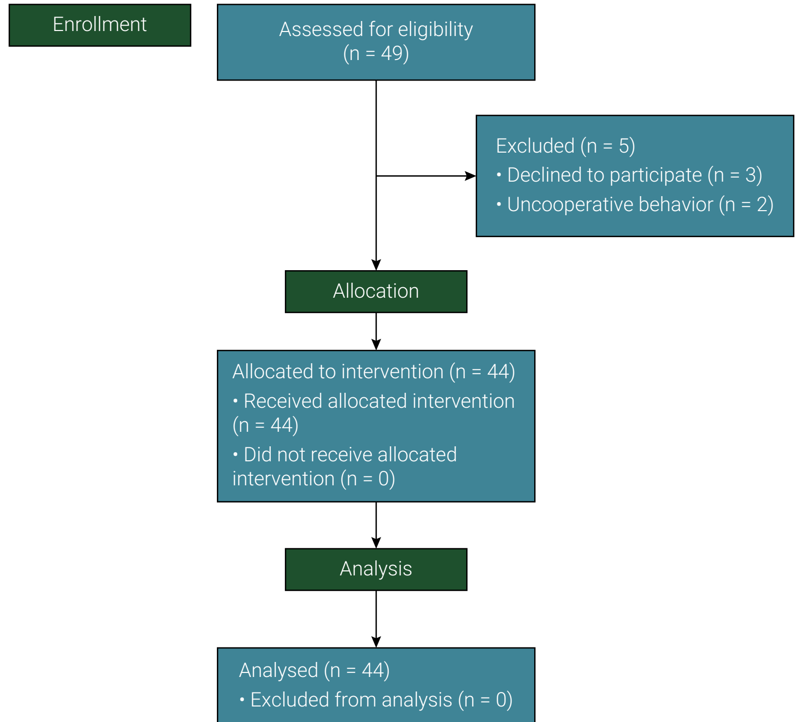


Figure 1. STROBE diagram of the patients' enrolment process.

Samples were collected from the root canal of deciduous teeth with primary endodontic infection of patients attending the Pediatric Dentistry Clinic of the Federal University of Ceara Dental School. Eligible participants were those presenting teeth with evidence of necrotic pulp tissue, less than two-thirds of root resorption, sufficient dental structure to permit full isolation with a rubber dam, and absence of periodontal pocket (>4mm). Moreover, they had to present no systemic alteration and used no antimicrobial medication within the past 3 months. Patients behaving uncooperatively were excluded from the study. Clinical and radiographic features were recorded. A specialist pediatric dentist performed the endodontic procedures and collected the samples.

The parents/caregivers were all informed about the study and signed a written informed consent form allowing their children to be recruited in it. The study was authorized by the Research Ethics Committee of the Federal University of Ceara, Fortaleza, CE, Brazil according to process number 224/10.

Microbiological Sampling

All clinical procedures of the microbiological sample collection have been previously described[18-20], but with some modifications. Briefly, after local anesthetic injection, the tooth was isolated with a rubber dam, and the operating area (rubber dam, clamp, and external area of the teeth) was disinfected with 30% hydrogen peroxide and 2.5% sodium hypochlorite before inactivation with 5% sodium thiosulfate. The disinfection of these sites was monitored by taking a swab from the external surfaces of the crown and surrounding areas. The swab was moistened into 50 µL of saline solution to facilitate the spreading of the content before streaking it onto a plate containing 5% defibrinated sheep blood and fastidious anaerobe agar (FAA, LAB M; Heywood, Lancashire, UK), which were then incubated anaerobically and aerobically, respectively, for up to 14 days. Next, DNA was extracted from the swab for polymerase chain reaction (PCR) analysis by using universal bacterial primers to confirm the disinfection.

High-speed spherical diamond burs (KG Sorensen, Sao Paulo, SP, Brazil) were used for accessing the pulp chamber under manual irrigation with sterile saline solution. Once the pulp chamber was accessed, it was irrigated with sterile saline. The microbiological sample collection was done immediately after these procedures, in which only one of the root canals was sampled. In the case of a multirooted tooth, the root canal with periapical radiolucency or the largest canal was chosen. Sterile absorbent paper points were inserted into the root canals up to 1 mm short of the apex and left for 60 seconds for sample collection. Next, the paper points were removed and stored in sterile Eppendorf tubes containing VMGA III transport medium at -80ºC.

DNA Extraction

DNA was extracted from the samples by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, for quantification of the DNA concentration (NanoDrop 2000; Thermo Scientific, Wilmington, DE, USA).

PCR Assay

The polymerase chain reaction (PCR) reaction was performed in a thermocycler (MyCycler; Bio-Rad, Hercules, CA, USA) at a total volume of 25 µL containing 2.5 µL 10× Taq buffer (1×), 0.5 µL dNTP mix (25 µmol/L of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 1.25 µL 25 mmol/L $MgCl_2$, 0.25 µL forward and reversal universal primers (0.2 µmol/L), 1.5 µL sample DNA (1 µg/50 µL), 1.5 µL Taq DNA polymerase (1 unit), and 17.25 µL nuclease-free water[21]. The primer sequences and their respective amplicon sizes are listed in Table 1. The target bacterial gene is located in the region of the 16S, which is specific for each species. The primers were synthesized by means of biosynthesis (Lewisville, TX, USA), and the PCR

products were examined by using 1% agarose gel electrophoresis under UV transillumination. Identification was positive or negative depending on the presence of clear bands of the expected molecular size by using a 21-kb lambda DNA ladder (Invitrogen Corporation, Carlsbad, CA, USA).

Table 1. PCR primers with expected amplicon sizes.

Target bacteria	Primers pairs (5' to 3')	Amplicon size (bp)	Reference
Actinomyces naeslundii	Forward: GCG CCT TTT TTG GTG TTT TTG G Reverse: CAC CCA CAA ACG AGG CAG GCC TG	274	(22)
Dialister pneumosintes	Forward: TTC TAA GCA TCG CAT GGT GC Reverse: GAT TTC GCT TCT CTT TGT TG	1105	(23)
Enterococcus faecalis	Forward: CCG AGT GCT TGC ACT CAA TTG G Reverse: CTC TTA TGC CAT GCG GCA TAA AC	138	(24,25)
Filifactor alocis	Forward: CAG GTG GTT TAA CAA GTT AGT GG Reverse: CTA AGT TGT CCT TAG CTG TCT CG	594	(24)
Fusobacterium nucleatum	Forward: AGT AGC ACA AGG GAG ATG TAT G Reverse: CAA GAA CTA CAA TAG AAC CTG A	1000	(24,25)
Parvimonas micra	Forward: AGA GTT TGA TCC TGG CTC AG Reverse: ATA TCA TGC GAT TCT GTG GTC TC	207	(23-25)
Porphyromonas endodontalis	Forward: GCT GCA GCT CAA CTG TAG TC Reverse: CCG CTT CAT GTC ACC ATG TC	672	(23-25)
Porphyromonas gingivalis	Forward: AGG CAG CTT GCC ATA CTG CG Reverse: ACT GTT AGC AAC TAC CGA TGT	404	(23-25)
Prevotella intermedia	Forward: TTT GTT GGG GAG TAA AGC GGG Reverse: TCA ACA TCT CTG TAT CCT GCG T	575	(23,24)
Prevotella nigrescens	Forward: ATG AAA CAA AGG TTT TCC GGT AAG Reverse: CCC ACG TCT CTG TGG GCT GCG A	804	(23,24)
Streptococcus mitis	Forward: GTC GAA GGT GAT GAT ATG AC Reverse: GAC AGT ACG CAG TCT TAC GTC	372	(26)
Streptococcus sanguis	Forward: GTC GAT GGC GAG GAT CTA GAG C Reverse: TGC CGA GCG CTC TAA CTC CA	208	(26)
Tannerella forsythia	Forward: GCG TAT GTA ACC TGC CCG CA Reverse: TGC TTC AGT GTC AGT TAT ACC T	641	(23-25)
Treponema denticola	Forward: TAA TAC CGA ATG TGC TCA TTT ACA T Reverse: TCA AAG AAG CAT TCC CTC TTC TTC TTA	316	(23-25)

Species-specific primers in the coding region of 16S rDNA were selected based on the previous investigation of endodontic bacteria by cloning and sequencing the bacterial 16S gene and on sequences available from GenBank. Species specificity was further confirmed by sequencing at least one PCR product from a clinical sample for a specific primer on an ABI Prism 310 automated sequencer (AME Bioscience Ltd, London UK), as previously described elsewhere[19].

Reference bacteria strains used in this study were acquired from the American Type Culture Collection (ATCC) and are listed as follows: *Actinomyces naeslundii* (ATCC 12104), *Dialister pneumosintes* (ATCC 33048), *Enterococcus faecalis* (ATCC 4083),

Filifactor alocis (ATCC 35896), *Fusobacterium nucleatum* (ATCC 25586), *Parvimonas micra* (ATCC 33270), *Porphyromonas endodontalis* (ATCC 35406), *Porphyromonas gingivalis* (ATCC 33277), *Prevotella intermedia* (ATCC 25611), *Prevotella nigrescens* (ATCC 33536), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguis* (ATCC10556), *Tannerella forsythia* (ATCC 43037), and *Treponema denticola* (ATCC 35405).

Checkerboard DNA–DNA Hybridization Assay

The presence and levels of bacterial species were examined by using checkerboard DNA–DNA hybridization[27]. All the 14 bacterial strains used in the preparation of DNA probes are presented in Table 2. Both PCR and checkerboard DNA–DNA hybridization used the same strains to allow comparison of the results.

Table 2. DNA probes used in the checkerboard DNA-DNA hybridization method for microbial characterization in root canals of deciduous teeth with primary endodontic infection

Microorganisms	ATCC
Actinomyces naeslundii	12104
Dialister pneumosintes	33048
Enterococcus faecalis	4083
Filifactor alocis	35896
Fusobacterium nucleatum sp. nucleatum	25586
Parvimonas micra	33270
Porphyromonas endodontalis	35406
Porphyromonas gingivalis	33277
Prevotella intermedia	25611
Prevotella nigrescens	33563
Streptococcus mitis	49456
Streptococcus sanguis	10556
Tannerella forsythia	43037
Treponema denticola	35405

Bacterial strains were grown anaerobically on the surface of blood agar plates (except two spirochetes, which were grown in broth) for 3 to 7 days, thus being harvested and placed in 1.5 ml microcentrifuge tubes containing 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at 3,500 rpm for 10 minutes. The cells were resuspended and lysed either using 10% SDS and proteinase K (20 mg/ml) (Sigma-Aldrich, St Louis, MO, USA) for Gram-negative strains or using 150 µl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma-Aldrich, St Louis, MO, USA) and 5 mg/ml of achromopeptidase (Sigma-Aldrich, St Louis, MO, USA) in TE buffer (pH 8.0) for Gram-positive strains. The pelleted cells were resuspended by means of sonication for 15 seconds and incubated at 37°C for 1 hour.

DNA was isolated and purified by using the method described by Smith et al.[28]. The concentration of purified DNA was determined by spectrophotometric measurement of absorbance at 260 nm. The purity of the preparations was assessed with a ratio of DNA to protein and a ratio of absorbance at 260 nm and 280 nm. Whole-genomic DNA probes were prepared for each of the 40 test strains by labeling 1 µg of DNA with digoxigenin (DIG DNA labeling kit, Sigma-Aldrich, St Louis, MO, USA) and using a random primer technique[29].

Briefly, the DNA was extracted by boiling and then fixed in a nylon membrane (Amersham Biosciences, Chicago, IL, USA) by using a Minislot 30TM apparatus (Immunetics, Cambridge, MA, USA). A Miniblotter 45TM (Immunetics Inc, Cambridge, MA, USA) device was used to hybridize DNA probes perpendicular to the lines of the clinical samples. Bound probes were detected by using a phosphatase-conjugated antibody to digoxigenin and chemiluminescence (CDP Star Detection Reagent™, Amersham Biosciences Corp., Amersham, UK).

Statistical Analysis

The comparison between the prevalence of specific pathogens measured by PCR and checkerboard DNA-DNA hybridization was statistically analyzed with Fisher's exact test. Sensitivity, specificity, and accuracy tests were performed. The statistical significance level was 5%.

Results

Forty-four root canals of deciduous teeth with primary endodontic infection were examined. Eligible children aged 2-9 years old were selected, with a mean age of 6 years old (±1), in which 21 (47.72%) were female and 23 (52.28%) male.

Traumatized anterior single-rooted teeth (n = 7) and necrotic posterior two-rooted or multi-rooted teeth (n = 37) were studied. With regard to the patients' clinical characteristics, none of them reported acute pain (acute abscess). However, 21 patients had sinus tract (chronic abscess). A detailed explanation of these clinical characteristics is shown in Table 3.

Table 3. Clinical and radiographic features of the patients according to the groups studied.

Variable	Category	Total
Gender	Female	21
	Male	23
Age	≥ 6 years	30
	< 6 years	14
Range of teeth	Single-rooted	7
	Bi-rooted	19
	Multi-rooted	18
Teeth localization	Upper	25
	Lower	19

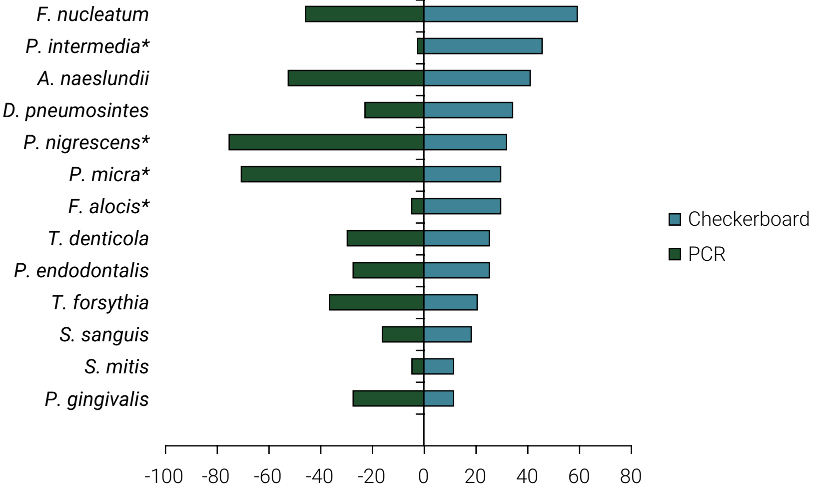
Continue

Continuation

Discoloration	Yes	7
	No	37
Pain on palpation	Yes	6
	No	38
Tenderness to percussion	Yes	6
	No	38
Sinus tract	Yes	21
	No	23
Mobility	Yes	1
	No	43
Periapical lesion ≤2mm	Yes	44
	No	0
Reason of infection	Caries	37
	Trauma	7

The presence of 14 target bacteria was investigated by using PCR and checkerboard DNA-DNA-hybridization.

All samples were found to contain the target bacteria, except *E. faecalis*, which was not found in the root canals by using both techniques, as shown in Figure 2. The results of PCR showed that the most frequently detected bacteria were *Prevotella nigrescens* (75%), *Parvimonas micra* (70.5%), *Actinomyces naeslundii* (52.3%), and *Fusobacterium nucleatum* (45.5%). The species *Prevotella intermedia* (2.3%), *Filifactor alocis* (4.5%), and *Streptococcus mitis* (4.5%) were found in less than 5% of the samples.



*There was a statistically significant difference between the prevalence of species detected by PCR and checkerboard (p<0.05).

Figure 2. Prevalence of specific endodontic pathogens detected by PCR and Checkerboard DNA-DNA hybridization in the root canal of deciduous teeth with primary endodontic infection.

The results of checkerboard DNA–DNA hybridization showed that the most frequently observed species were *F. nucleatum* (59.1%), *P. intermedia* (45.5%), and *A. naeslundii* (40.9%). *Porphyromonas gingivalis* (11.4%), *S. mitis* (11.4%), and *Streptococcus sanguis* (18.2%) were found in less than 20% of the samples. Figure 3 shows the levels of each species.

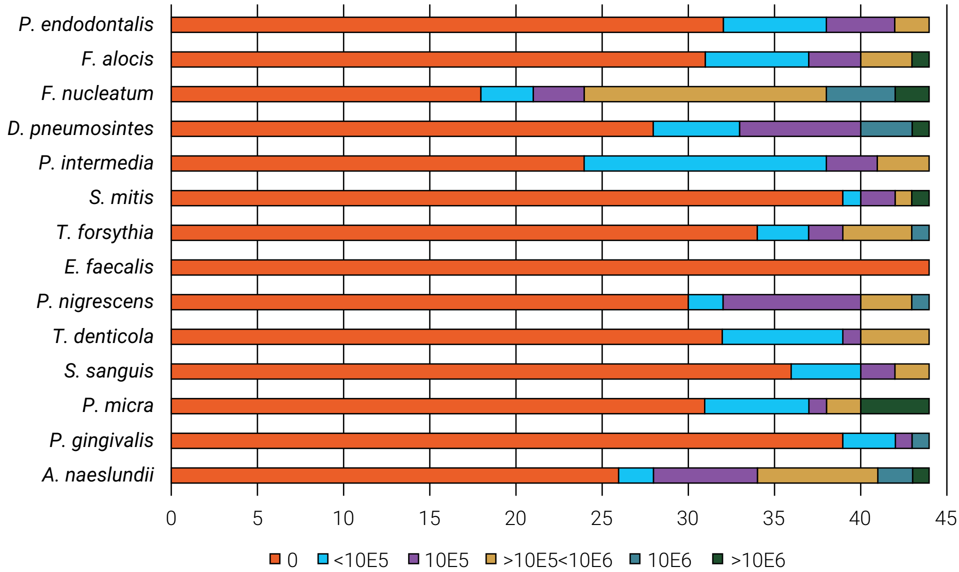


Figure 3. Checkerboard results show the levels of each species found in the 44 samples.

A. naeslundii and *F. nucleatum* were the most prevalent species, taking together the results from both methods. *A. naeslundii* was detected by PCR in 23 (52.3%) and by checkerboard DNA–DNA hybridization in 18 (40.9%) ones, with 10 (22.7%) having matched positive results. *Fusobacterium nucleatum* was identified in 20 (45.5%) cases by PCR and 26 (59.1%) ones by checkerboard DNA–DNA hybridization, with 11 (25.0%) having matched positive results. On the other hand, *P. nigrescens* and *P. micra* were more prevalent when examined by PCR (75% and 70.5%, respectively) than by checkerboard DNA–DNA hybridization (31.8% and 29.5%, respectively). Matching positive results were found for 12 and 10 cases, respectively. *Prevotella intermedia* and *F. alocis* were more prevalent when examined by checkerboard DNA–DNA hybridization (45.5% and 29.5%, respectively) than by PCR (2.3% and 4.6%, respectively). No matching positive results occurred for *P. intermedia* and just one matching positive result was found for *F. alocis*.

There was a significant difference between PCR and checkerboard DNA–DNA hybridization concerning the detection of specific endodontic pathogens. PCR demonstrated a significantly higher prevalence of *P. micra* ($P < 0.05$) and *P. nigrescens* ($P < 0.05$) compared to checkerboard DNA–DNA hybridization, which in turn showed a significantly higher prevalence of *F. alocis* ($P < 0.05$) and *P. intermedia* ($P < 0.05$) com-

pared to PCR. It was not observed any statistically significant difference between the frequencies of the other species analyzed by both methods.

Table 4 shows the matching positive results, sensitivity, specificity, and accuracy for PCR and checkerboard DNA–DNA hybridization according to statistical tests. Checkerboard DNA–DNA hybridization had a sensitivity ranging from 0 to 0.86, specificity from 0.30 to 0.97, and accuracy from 0.11 to 0.68, in which sensitivity was higher for *P. nigrescens*, specificity was higher for *F. alocis* and accuracy was higher for *P. nigrescens* and *T. forsythia*. PCR had a sensitivity ranging from 0 to 0.55, specificity from 0.38 to 0.94, and accuracy from 0.01 to 0.70, in which sensitivity was higher for *F. nucleatum*, specificity was higher for *P. gingivalis* and accuracy was higher for *P. nigrescens*.

Table 4. Matching positive results, sensitivity, specificity, and accuracy of endodontic pathogens detected by PCR and Checkerboard DNA-DNA hybridization in the root canal of deciduous teeth with primary endodontic infection.

	Matching positive results n (%)	Sensitivity		Specificity		Accuracy	
		Checker-board	PCR	Checker-board	PCR	Checker-board	PCR
A. naeslundii	10 (22.7)	0.56	0.43	0.50	0.62	0.53	0.53
D. pneumosintes	2 (4.5)	0.13	0.20	0.72	0.62	0.33	0.29
F. alocis	1 (2.3)	0.08	0.50	0.97	0.71	0.34	0.51
F. nucleatum	11 (25.0)	0.42	0.55	0.50	0.38	0.47	0.47
P. micra	10 (22.7)	0.77	0.32	0.32	0.77	0.64	0.64
P. endodontalis	3 (6.8)	0.27	0.25	0.73	0.75	0.39	0.39
P. gingivalis	3 (6.8)	0.60	0.25	0.77	0.94	0.62	0.44
P. intermedia	0 (0)	0	0	0.96	0.53	0.44	0.01
P. nigrescens	12 (27.3)	0.86	0.36	0.30	0.82	0.68	0.70
S. mitis	0 (0)	0	0	0.95	0.88	0.11	0.04
S. sanguis	3 (6.8)	0.38	0.43	0.89	0.86	0.47	0.50
T. forsythia	6 (13.6)	0.67	0.38	0.71	0.89	0.68	0.56
T. denticola	2 (4.5)	0.18	0.15	0.67	0.71	0.30	0.32

Discussion

Molecular biology has permitted the detection and identification of a great number of species by allowing an improved interpretation of the etiopathogenesis of endodontic infections[10]. When comparing PCR and checkerboard DNA–DNA hybridization to detect endodontic pathogens, our results have shown that both methodologies presented different results.

Sensitivity, specificity, and accuracy are generally terms used in statistics to assess a diagnostic test. Sensitivity is defined as the proportion of individuals with

a condition who will have a positive result[30]. The result should be considered true positive when the condition is present and the diagnostic test is positive too. It demonstrates how effective the test is at detecting a condition. Mathematical upsides of sensitivity address the likelihood of a diagnostic test determining patients who actually have the disease. The higher the value of sensitivity, the less likely the chance of a diagnostic test yielding a false-positive result[31]. In our study, checkerboard DNA–DNA hybridization had a higher sensitivity to detect *P. micra* and *P. nigrescens*, whereas PCR had a higher sensitivity to detect *F. alocis* and *F. nucleatum*. These species have been related to the most frequent species isolated from primary endodontic infection of permanent teeth[18,20,32]. *Prevotella nigrescens* and *F. nucleatum* were most frequently detected by studies using PCR[33] and checkerboard DNA–DNA hybridization[6,7] to investigate the presence of endodontic pathogens in primary teeth.

Specificity is the proportion of individuals without a condition who will have a negative result[27]. The test outcome is genuinely negative when the condition is demonstrated to be absent and when the diagnostic test shows that the condition is also negative, thus indicating how effective the test is at determining this negativity. Numerically, specificity represents the probability of a disease being diagnosed without false-positive results[31].

In our study, PCR and checkerboard DNA–DNA hybridization had high specificity for the majority of the species analysed. We observed that specificity showed better results than sensitivity. For the sensitivity results, the checkerboard DNA–DNA hybridization was slightly better than PCR. According to Siqueira et al.[17], the positive results of PCR-positive and checkerboard DNA–DNA hybridization may be due to a cross-reactivity for the whole DNA probe. PCR might have recognized microorganisms with genetic similarities to the wanted species differentiated by a small number of nucleotides, which would not be likely detected by 16S rRNA investigation.

Accuracy is the ratio of genuine results, whether it is positive or negative, in a population. It assesses the level of authenticity of a diagnostic test and how accurately the test confirms and rejects a condition[31]. Besides accuracy being determined with sensitivity and specificity, it is also based on prevalence. Therefore, even if sensitivity and specificity have a high value, the accuracy is not essentially high[30]. In our study, higher accuracy values were found for *P. nigrescens* by both PCR and checkerboard DNA–DNA hybridization. This result indicates how common this pathogen is in the selected samples, which is corroborated by other studies[6,7,18,20,32,33].

In our literature search, we found just one study comparing sensitivity, accuracy, and specificity of PCR to those of checkerboard DNA–DNA hybridization to detect microorganisms present in root canals of adult patients[17]. According to these authors, both methods were similar regarding this detection. In the present study, PCR and checkerboard DNA–DNA hybridization were somewhat in accordance and revealed an acceptable number of identical results for most target microorganisms, except that the prevalence of *P. endodontalis* and *T. denticola* were significantly higher by the former than by the latter assay.

PCR and checkerboard DNA-DNA hybridization had specificity higher than sensitivity, demonstrating that these assays can identify true negatives in the samples, thus corroborating the literature[17]. Both methods showed a similar capacity to detect specific endodontic pathogens in root canals of deciduous teeth with primary endodontic infection. These methods showed no significant differences in the prevalence of most species detected, except for four species, in which PCR showed more prevalence of *P. micra* and *P. nigrescens* and checkerboard DNA-DNA showed more prevalence of *F. alocis* and *P. intermedia*. Interestingly, *Enterococcus faecalis*, a commonly found microorganism in the root canals of deciduous[17] or permanent teeth[34], was not detected by either technique. Similarly, Tavares et al.[6] reported its low prevalence (3.2%) alongside *Eikenella corrodens* (3.1%).

One limitation of this study is that during the use of checkerboard DNA-DNA hybridization with whole DNA probes, there is a risk of cross-reactivity between species due to the presence of homologous sequences between different bacterial species[17,35]. However, it is important to highlight that the prevalence of cross-reaction tests was shown to be low between different species of the same genera, with virtually no cross-reaction being observed in previous studies[27,36]. In addition, PCR and checkerboard DNA-DNA hybridization use a different amount of DNA and this might have influenced the results[17].

PCR, known for its high sensitivity and specificity, enables the rapid and precise identification of bacterial DNA at low concentrations, making it invaluable in clinical diagnostics[10,37]. However, its high sensitivity makes it susceptible to contamination, resulting in potential false positives, and it only provides prevalence results[10,37]. In contrast, checkerboard DNA-DNA hybridization, while generally less sensitive and specific than PCR, has the advantage of high throughput, allowing for the simultaneous analysis of multiple bacterial species across multiple samples as well as the quantification of species levels[10,15]. This method is inexpensive for large-scale research and has well-established protocols for analyzing complex microbial communities, particularly in the oral microbiome[10,15]. Despite being labor-intensive and less precise in quantification, checkerboard DNA-DNA hybridization is an effective tool for conducting comprehensive epidemiological studies[10,15]. Therefore, the specific needs of the research or clinical application should guide the choice between these techniques, balancing sensitivity, specificity, throughput, and cost.

In conclusion, both methods are efficient in detecting endodontic pathogens and one should complement the other. Moreover, taking together their results, *A. naeslundii* and *F. nucleatum* were the most prevalent species.

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Conflict of Interest

The authors have no conflict of interest to disclose.

Data availability

Datasets related to this article will be available to the corresponding author upon request.

Author Contribution

Denise Lins de Sousa: conceived and designed the experiments, performed the laboratory experiments, wrote the manuscript. **Rebecca Bastos Rocha Araújo:** conceived and designed the experiments, performed the laboratory experiments, wrote the manuscript. **Thaís Mageste Duque:** conceived and designed the experiments, performed the laboratory experiments. **Juliana Delatorre Bronzato:** conceived and designed the experiments, performed the laboratory experiments, wrote the manuscript. **Magda Feres:** conceived and designed the experiments, analyzed the data and reviewed the writing of the manuscript. **Brenda Paula Figueiredo de Almeida Gomes:** conceived and designed the experiments, analyzed the data and reviewed the writing of the manuscript, wrote the manuscript. **Juliana Oliveira Gondim:** conceived and designed the experiments, analyzed the data and reviewed the writing of the manuscript. **José Jeová Siebra Moreira-Neto:** conceived and designed the experiments, analyzed the data and reviewed the writing of the manuscript, wrote the manuscript. All authors actively revised and approved the final version of the manuscript.

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