

Full Length Research Paper

Detection of *Bacteroides forsythus* and *Porphyromonas gingivalis* in infected root canals during periapical periodontitis by 16S rDNA

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Periapical periodontitis is termed when inflammation of the periodontium is caused by irritants of endodontic origin. Bacterial strains in the root canals were not easy to be identified by the traditional agar culture. In this study a 16S rDNA-based polymerase chain reaction detection method was used to determine the occurrence of *Bacteroides forsythus* and *Porphyromonas gingivalis* in chronic periapical periodontitis among Chinese patients. 217 patients with chronic periapical periodontitis were recruited and a total of 266 teeth were collected. The subjects had no systemic diseases, no antibiotics taken, no root canal treatment (RCT) performed on the infected teeth in the last 3 months. The DNA of bacteria in the root canal was extracted and amplified using universal 16S rDNA primers. The amplification was performed to detect *B. forsythus* and *P. gingivalis* using oligonucleotide primers designed from species-specific 16S rDNA signature sequences. *B. forsythus* and *P. gingivalis* were detected in 26 and 40% of the participants, respectively. 24 out of 217 infected root canals demonstrated the existence of both types of bacteria, the utility of a 16S rDNA-based PCR detection method showed high sensitivity and high specificity to directly detect *B. forsythus*, *P. gingivalis* or other pulpal microorganisms from samples of root canal infections. The results indicated that *B. forsythus* or *P. gingivalis* might be a member of the microbiota associated with chronic periapical periodontitis and there was a strong association between the studied species and periodontitis.

Key words: *Bacteroides forsythus*, *Porphyromonas gingivalis*, chronic periapical periodontitis, 16S rDNA, polymerase chain reaction.

INTRODUCTION

Periapical periodontitis is termed when inflammation of the periodontium is caused by irritants of endodontic origin (Cohen and Burins, 2002; Haapasalo, 1989; Munson et al., 2002; Sundqvist et al., 1989). In the root canals of chronic apical periodontal teeth, 3 to 12 strains of bacteria could be found with colony forming units between 10^2 and 10^8 . These consisted predominantly of strict anaerobes, such as black-pigmented bacteria, *Peptostreptococci*, *Eubacterium spp*, *Fusobacterium nucleatum* and *Actinomyces spp* (Brook et al., 1996; Gomes et al., 1994, 1996; Yoshida, 1987). However, the

role of any particular strains of micro-organisms in the pathogenesis of periapical periodontitis still remains uncertain and is being investigated.

The black-pigmented bacteria, a strains of the normal oral flora, includes *Prevotella spp* and *Porphyromonas*, of which *Porphyromonas endodontalis* and *Porphyromonas gingivalis* are controversial in their pathogenicity in the oral cavity. Some workers believed that adult periodontitis is caused mainly by *P. gingivalis* whereas *P. endodontalis* causes periapical diseases (Shah et al., 1998; Lopes and Siqueira, 2004). *P. gingivalis* is undeniably involved in periodontal diseases since it is found in infected root canals whether by separate colony culture or polymerase chain reaction (PCR). Nevertheless, collagenase PrtC was detected in the cell line cultured from *P. gingivalis* found in root canal (Beikler et al., 2003;

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Bodinka et al.,1994; Kato et al.,1992; Takahashi et al., 1991) but this was absent in *P. endodontalis* (Odell et al.,1999). Therefore, it is worth exploring further about the effects of *P. gingivalis* on periapical pathologies.

In general, anaerobic bacterial strains in the root canals are not easily identified by traditional agar culture. *Bacteroides forsythus* was thought to be the causal bacteria for periodontitis but its culture from infected root canals has retrieved no success thus far. Therefore, there is a need to make use of a more advanced technique to monitor the microbial environment in the pulp. PCR method is the best choice since it has a higher sensitivity and specificity on identification of bacteria than the traditional bacterial culture.

In this study, the existence of *B. acteroides forsythus* and *P. gingivalis* in the root canal, as well as the relationship between the two were examined. The association of these bacteria on the inflammation of periodontal tissues, causing chronic periapical periodontitis among a group of Chinese patients was also determined.

METHODS AND MATERIALS

Subject selection

217 patients (147 males and 70 females) with chronic periapical periodontitis were recruited in Jinhua Dentistry, Chengdu for this study with their consent. The research protocols were approved by the ethics committee of Sichuan University. The patients were aged between 11 and 73 with the mean age of 48. Some of their medical history and clinical symptoms of affected teeth were obtained, which included spontaneous pain, drugs prescribed, root canal treatment (RCT), etc. A total of 266 teeth were collected. The check-up criteria comprised pulp exposure, percussion pain, sinus tract and pus discharge, probe depth and periapical radiolucency of the infected tooth. The subject selection criteria was that they should be free of systemic diseases, no antibiotics taken, no RCT performed on the infected teeth in the last 3 months, no radiolucencies observed on radiographs. The collected teeth were divided into 2 groups depending on the exposure of pulp. There were 56 teeth with pulp exposure whereas the other 210 without.

Sample collection

The extracted teeth were rinsed with 2% chlorhexidine followed by 0.9% normal saline. Plaque and calculus were removed from the tooth surfaces which were then cleansed with 3% H₂O₂. Sterilization was performed using iodine followed by 2.5% sodium hypochlorite (NaOCl) to dissolve any organic materials that were left on the tooth surfaces. The teeth were dried by compressed air. A sterile high-speed handpiece and bur were used to drill into the top of the pulp and any fillings were removed in the presence of water coolant. The cavity was sterilized by the same method as for tooth surfaces. The pulp chamber was drilled open by carbide round bur on a low-speed handpiece without water coolant (Jung et al., 2001; Slots and Taubman, 1992).

An electronic root canal measuring device (Root ZX, J. Morita Mfg. Co., Kyoto, Japan) was chosen for endodontic working length determination. However, if pus was present in the root canal, the working length determination would be based on radiographs, palpation and patient's response. K-type file sized 15 was used to

scrape the root canal wall in a linear motion according to the recorded working length. Sterile tiny paper points were placed into the root canal for 30 s to collect fluid and this was repeated three times. If no fluid was detected, a suitable amount of 0.9% sterile saline was added to the root canal to dissolve any bacteria into the fluid. The paper points were immersed in a clean eppendorf tube containing 1ml phosphate buffered saline (PBS) to be stored at -20°C.

DNA isolation

The DNA of the bacteria in the root canal fluid was extracted. 25% Chelex100 (Bio-Rad, USA) was added to the sample in a 1:3 fashion. The bacterial DNA was released into the solution by incubation in 56°C for 30 min followed by 100°C for ten minutes. After centrifugation at 15000 rpm for five minutes at 4°C, the DNA in the supernatant was placed in -20°C for storage (Siqueira et al., 2008).

Primer design and PCR amplification of 16S rDNA

For the detection of *B. forsythus*, the primers (Shenergy biocolor life science Co., Ltd, Shanghai, RPC) used for the PCR amplification of 16S rDNA sequences were (forward) 5'-GCGTATGTAACCTGCCCGCA-3' and (reverse) 5'-TGCTTAAGTGTGTCAGTTATACCT-3' (Dix et al., 1990). The primer sequences of *P. gingivalis* were (forward) 5'-TGTAGATGACTAATGGTGAAAACC-3' and (reverse) 5'-ACGTCATCCCCACCTTCCTC-3' (Rumpf et al., 1999). The expected sizes of PCR products obtained with these primers were 641 and 197 bp, respectively. A positive control using a universal primer pair was also included in each experiment: (forward) 5'-GATTAGATACCCTGGTAGTCCAC-3' and (reverse) 5'-CCCGGAACGTATTCACCG-3' (McClellan et al., 1996). Its expected size of PCR product was 602 bp. PCR was performed by DNA thermal cycler (Eppendorf-Netheler-Hinz GmbH 22331, Hamburg, Germany). 4 µl of template DNA was amplified by Takara Taq (Takara biotechnology, Dalian, China) preceded by addition to a final volume of 50 µl containing, 5µl of 10 x Taq buffer, 5 µl 25 mM MgCl₂, 4 µl 2 mM each deoxynucleoside triphosphate (dNTP), 1.5 U of Taq DNA polymerase, 1.6 µM forward primer and 1.6 µM reverse primer. The amplification of PCR was used in an initial denaturation at 95°C for min, 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min and a single final extension at 72°C for 7 min. The purity of the product was determined by electrophoresis in a 1.5% agarose gel. DNA was stained with ethidium bromide and the gels were captured in imaging system (Dolphin-Doc, Wealtec, NV, USA).

Statistical analysis

The clinical and experimental data were collected for each case and were statistically analyzed (15.0 SPSS IL, USA) to determine the relationship between the presence of *B. forsythus*, *P. gingivalis*, the infected, and non-exposed root canal (Fisher's exact test) ($P > 0.05$). If there was a significant difference, the odds ratio was calculated. According to the method of standard determination by Socransky et al. (1988) and Sundqvist (1993), positive associations were those with an odds ratio > 2 and negative associations were those with an odds ratio < 0.5 . Using OR 95% confidence interval, when $OR < 1$ in positive association or when $OR > 1$ in negative association, it was considered to be no statistical significances.

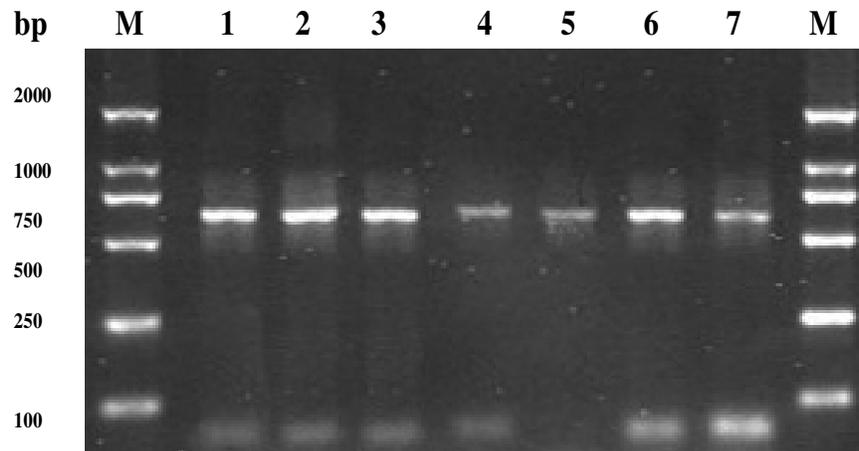


Figure 1. 602 bp PCR products were identified in all samples in 1.5% agarose gel electrophoresis. M stands for molecular weight of markers (100 bp DNA). Lanes 1 - 7 were various samples of the infected root canal. The lowest band appeared in lane M was 100 bp.

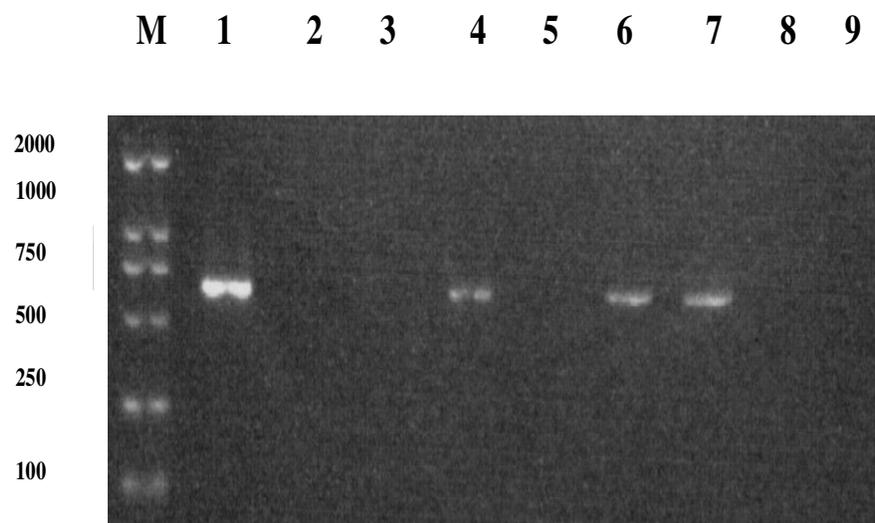


Figure 2. 641 bp PCR products of 16S rDNA in *B. forsythus* were visualized by 1.5% agarose gel electrophoresis in some samples. M stands for molecular weight markers (100 bp DNA). Lanes 1-9 were various samples of infected root canal. The lowest band appeared in lane M was 100-bp.

RESULTS

Evaluation of *B. forsythus* and *P. gingivalis* specific primers

With a universal primer pair, a 602 bp PCR-product was amplified in all samples (Figure 1). Some samples demonstrated the presence of *B. forsythus* by a 641 bp PCR-product (Figure 2). On the other hand, using the universal primer, a 197 bp PCR-product was amplified. The detection of *P. gingivalis* with a PCR-product of 197 bp was shown in some samples (Figure 3 and 4).

The relationship between *B. forsythus*, *P. gingivalis* and periodontitis

Out of 266 infected teeth collected, 119 teeth (44.7%) had been identified with the presence of either of the two bacteria (Table 1). 70 teeth (26.3%) were marked with the occurrence of *B. forsythus* while *P. gingivalis* were found in 105 teeth (39.5%). *B. forsythus* and *P. gingivalis* co-existed in 56 teeth (21.1%). The OR value was 12 which indicated that there was a direct relationship between the 2 bacteria and chronic periapical periodontitis.

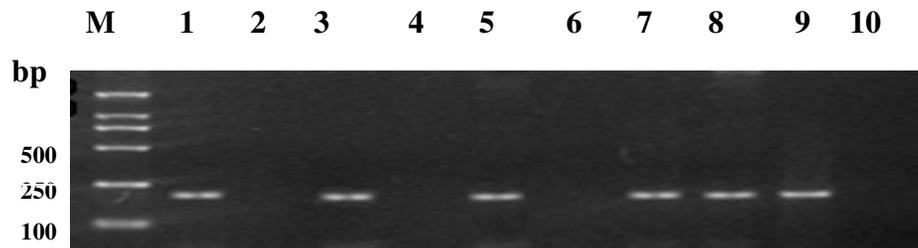


Figure 3. 197bp PCR products of 16S rDNA in *P. gingivalis* were visualized by 1.5% agarose gel electrophoresis in some samples. M stands for molecular weight markers (100 bp DNA). Lanes 1-9 were various samples of infected root canal. The lowest band appeared in lane M was 100-bp.

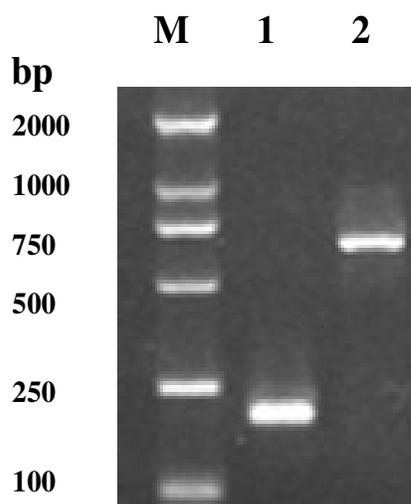


Figure 4. Agarose gel electrophoresis demonstrating the existence of both *B. forsythus* at 641 bp and *P. gingivalis* at 197 bp in the root canal of a mandibular canine.

The effect of pulp exposure to the colonization of *B. forsythus* and *P. gingivalis*

Among the 210 teeth with intact pulp, there were 63 teeth (30.0%) marked with the presence of *B. forsythus* (Table 2) while there was 7 out of 56 teeth (12.5%) with pulp exposure had *B. forsythus*. No statistical significance was shown between the exposure of pulp cavity and the presence of *B. forsythus*. The OR value between the colonization of *B. forsythus* in infected root canal and intact pulp was 3, which was larger than 2, indicating that *B. forsythus* had a positive association between these two parameters.

There were 91 out of 210 teeth (43.3%) marked with the presences of *P. gingivalis* in intact pulp (Table 2) while 14 out of 56 teeth (25.0%) had *P. gingivalis* presented under the exposure of pulp. A lack of relationship was observed between the exposure of pulp cavity and the presence of *P. gingivalis*. The OR value between the colonization of *P. gingivalis* in root canal and intact pulp

was 2.29, which was larger than 2, indicating that *P. gingivalis* had a positive association between these two parameters.

Meanwhile, 7 of out 56 teeth (12.5%) (Table 2) with pulp exposure had been detected with the presence of both bacteria, *B. forsythus* and *P. gingivalis*, while 49 out of 210 teeth (23.3%) had been detected with the presence of both bacteria with intact pulp.

DISSUSSION

To detect and identify bacteria in the root canal, bacterial cultivation method had been widely used and had been considered as a gold standard to identify bacteria. Theoretically, with the presence of only one bacterium, this method can already detect its occurrence. However, there are 2 prerequisites - a certain amount of living bacteria must be present and that the bacteria must be able to grow, proliferate, easily isolated, and identified, on an artificially made culture medium in laboratory before being monitored and tested. Loesche et al. (1992) reported that the minimum amount of living bacteria of one species was 2×10^3 cells in order to be identified by bacterial cultivation method. Zambon and Haraszthy (1995) revealed that the minimum living bacteria in non-selective cultural medium to be between 10^4 - 10^5 and 10^3 in selective culture medium. Also, a report showed that the minimum living bacteria to colonize the pulp was 5×10^1 when liquid medium was used for culture (Moller, 1966). Chronic periapical periodontitis is a disease caused by infections of certain bacteria. Using traditional bacterial cultivation not only requires thousands or 100,000 of living bacteria, it also requires ideal environments for cultivation such as culture medium, anaerobic condition, etc. Therefore, 16S rDNA PCR method is preferred over bacterial cultivation.

16S rDNA PCR was used in this experiment for the detection of *B. forsythus* and *P. gingivalis*. This is a highly sensitive method as it can detect DNA from living bacteria. It can also detect non-degraded DNA from dead bacterial cells. As a result, the specific gene sequences

Table 1. The relationship of the prevalence of *B. forsythus* and *P. gingivalis* in the root canal specimens.

		<i>B. forsythus</i>		Total
		Positive	Negative	
<i>P. gingivalis</i>	Positive	56 (21.1%)	49 (18.4%)	105 (39.5%)
	Negative	14 (5.3%)	147 (55.3%)	161 (60.5%)
	Total	70 (26.3%)	196 (73.7%)	266 (100%)

OR = 12, *P < 0.05.

Table 2. The relationship of pulp exposure and the presence of *B. forsythus* and *P. gingivalis* in the chronic periapical periodontitic root canal specimens.

Parameter	<i>B. forsythus</i> (%)	<i>P. gingivalis</i> (%)	<i>B. forsythus</i> and <i>P. gingivalis</i> (%)
Pulp exposure 56 cases	7 (12.5)	14 (25.0)	7 (12.5)
Intact pulp 210 cases	63 (30.0)	91 (43.3)	49 (23.3)
Total 266 cases	70 (26.3)	105 (39.5)	56 (21.1)

of different bacteria were amplified for 10^7 times. As suggested by other reports (Ashimoto et al., 1996; Bogen and Slots, 1999; Molander et al., 2002; Zambon and Haraszthy, 1995), small amount of bacterial genes could still be detected precisely by the method of 16S PCR. Having only 5 - 1000 bacterial cells or 10 – 13 g of total DNA in around 20 bacterial genes in our experiment, these were enough to be detected by 16S PCR.

The presence of *B. Forsythus* and *P. gingivalis* was consistent with Ximenz-Fyvie's report (Ximenez-Fyvie et al., 2000). Our results indicated that among the 266 infected teeth with chronic periapical periodontitis, 26.3% of the teeth were identified with *B. forsythus*, and 39.5% with *P. gingivalis* while both results were lower than that of non-infected teeth.

B. Forsythus has long been suspected to cause chronic periapical periodontitis. However, this bacterium was very difficult to culture, purified and identified using the traditional bacterial cultivation method. Furthermore, reports published in the last decade did not classify *B. Forsythus* as a micro-organism that caused infection of root canal and fewer reports have indicated the relationship between dental pulp infections and periapical periodontitis. Surprisingly, since the introduction of techniques in molecular biology and dental microbiology, bacteria that were used to be very difficult to cultivate have been identified (Conrads et al., 1997, Goncalves and Mouton, 1999, Jung et al., 2001, Rôças et al., 2001; Siqueira et al., 2000; Sunde et al., 2000).

Meanwhile, *P. gingivalis* was detected in 105 teeth out of 266 (39.5%), which indicated that this bacterium is associated with root canal infection in chronic periapical periodontitis. It was consistent with suggestion that black pigmented *Bacteroides* like *Porphyromonas* (*P. gingivalis*, *P. endodontalis*) and *Prevotella* (*P. intermedia*) played an important role in the infection of root canal (Slots and

Taubman, 1992). With the employment of 16S PCR, *P. gingivalis* was discovered to be able to colonize inside root canal.

It was suggested that by our experimental result that the two bacteria, *B. Forsythus* and *P. gingivalis*, have mutual coexistence inside the root canal. OR value and statistical analysis revealed that there was a positive association between the colonization of *B. Forsythus* and *P. gingivalis* in the root canal of the infected teeth. This was also identified by other reports (Rôças et al., 2001, Socransky and Haffajee, 1997) and suggested that there was a mutual relationship between these two species. It has been reported that *B. Forsythus* alone did not cause infection and inflammation, but when it was mixed with other species of bacteria, it induced inflammation (Takemota et al., 1997). Similarly, some reports suggested that *P. gingivalis* alone mostly would not cause infection, but only when mixed with other bacteria, it would cause inflammation (Sundqvist, 1993; Siqueira et al., 1998). These revealed that cross infection of *B. Forsythus* and *P. gingivalis* had greatly increased the toxicity and infectivity of chronic periapical periodontitis.

In conclusion, our experiment successfully detected the presence of 2 bacterial strains, *B. Forsythus* and *P. gingivalis* using 16S PCR method. Both bacterial strains had a direct relationship between their existences and chronic periapical periodontitis. There was no statistical significance to support the relationship between the intact pulp cavity and the exposure of pulp cavity on the colonization of these 2 bacteria. 16S PCR method is quick and reliable in detecting bacteria from samples of dental pulp or periodontal tissues *in vitro*.

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