Campylobacter gracilis and *Campylobacter rectus* in primary endodontic infections

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Abstract

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Aim A species-specific nested polymerase chain reaction (PCR) assay was used to investigate the occurrence of *Campylobacter gracilis* and *C. rectus* in primary root canal infections.

Methodology Samples were collected from 57 single-rooted teeth with carious lesions, necrotic pulps and radiographic evidence of periradicular disease. Twenty-eight cases were diagnosed as chronic asymptomatic periradicular lesions, 12 cases as acute apical periodontitis, and 17 cases as acute periradicular abscess. DNA was extracted from the samples and initially amplified using universal 16S rDNA primers. A second round of amplification using the first PCR products was performed to specifically detect *C. gracilis* or *C. rectus* in the samples.

Results *Campylobacter gracilis* and *C. rectus* were, respectively, detected in 21.4 (6 of 28) and 30% (6 of 20) of the root canals associated with chronic asymptomatic periradicular lesions. *Campylobacter gracilis* was found in 16.7% (2 of 12) of the cases diagnosed as acute apical periodontitis, whilst *C. rectus* was found in 33.3% (two of six cases). In the abscessed cases, *C. gracilis* and *C. rectus* were detected in 23.5 (4 of 17) and 11.8% (2 of 17) of the cases, respectively. No association of these species with clinical symptoms was observed ($P > 0.01$). In general, species-specific nPCR allowed the detection of *C. gracilis* in 21.1% (12 of 57) and *C. rectus* in 23.3% (10 of 43) of the samples taken from primary endodontic infections.

Conclusions Findings confirmed the assertion that both *C. gracilis* and *C. rectus* participate in infections of endodontic origin and suggest a pathogenetic role with regard to periradicular diseases.

Keywords: *Campylobacter gracilis*, *Campylobacter rectus*, endodontic infections, nested PCR.

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Introduction

Primary endodontic infection is caused by microorganisms colonizing the necrotic pulp tissue. In general, primary infections are mixed and predominated by anaerobic bacteria. Although more than 200 microbial species have been isolated/detected from infections of endodontic origin, a more restricted group composed of 15–30 species has been implicated as candidate endodontic pathogens (Siqueira 2002). Such putative pathogens belong to the genera *Treponema*, *Bacteroides*, *Porphyromonas*, *Prevotella*, *Fusobacterium*, *Peptostreptococcus*, *Eubacterium* and *Campylobacter*.

Campylobacter gracilis is a nonmotile, nonspore forming, anaerobic Gram-negative rod with a formate- and fumarate-requiring metabolism. Cells are small and straight, 0.4 μm wide and 4–6 μm long, with round ends. The G + C content of the DNA is 43–47 mol% (Tanner *et al.* 1981, 1992). This species was proposed and described by Tanner *et al.* (1981), as *Bacteroides gracilis*. Vandamme *et al.* (1995) analysed the cellular fatty acids, respiratory quinones and proteins of *B. gracilis*, and compared the features with the corresponding chemotaxonomic features of its closest relatives, the Campylobacters. Their results and previously published data for genotypic and phenotypic characteristics were used to

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reconsider the classification of this species, transferring it to the genus *Campylobacter*.

Campylobacter rectus is a small, nonspore forming, asaccharolytic, microaerophilic Gram-negative rod capable of motility via a single polar flagellum. Cells are frequently straight, 0.5 μm wide and 4 μm long, but may occasionally appear curved or helical. Regarding its metabolism, formate or hydrogen from several oral microorganisms serves as electron donors, whilst nitrate or fumarate from arpartate-producing microorganisms serves as electron acceptors. The G + C content of the DNA is 42–46 mol% (Tanner *et al*. 1981, 1992). The species was described by Tanner *et al*. (1981), as *Wolinella recta*, and further transferred to the genus *Campylobacter* (Vandamme *et al*. 1991).

Campylobacter gracilis and *C. rectus* have been recovered from different forms of periodontal diseases and claimed to have a potential pathogenic role in such diseases (Rams *et al*. 1993, Tanner *et al*. 1997, 1998, Kamma *et al*. 2000, Macuch & Tanner 2000). Studies have also isolated/detected these *Campylobacter* species from endodontic infections in variable prevalence values (Ranta *et al*. 1988, Sundqvist *et al*. 1989, 1998, Sundqvist 1992, Gomes *et al*. 1996, Le Goff *et al*. 1997, Siqueira *et al*. 2000b, 2001b).

Because these species are not always easily identified by conventional phenotype-based identification procedures, it is possible that their prevalence has been underestimated in primary endodontic infections. Molecular technologies, particularly the polymerase chain reaction (PCR) method, overcome many of the problems associated with traditional phenotype-based identification methods. PCR has been widely used to identify microbial species that are difficult or impossible to cultivate, and strains difficult to identify due to a phenotypically convergent or divergent behaviour (Relman 1993, 1999). The PCR methodology has the highest detection rate between the microbiological identification methods, and under optimized conditions also shows high specificity (McPherson & Moller 2000). The nested PCR (nPCR) technique is a modification of the PCR technology that involves a first amplification reaction of a DNA sequence with one set of primers followed by reamplification using a second set of primers complementary to smaller specific sequences within the first PCR product. nPCR can show increased sensitivity and even improved specificity when compared with single PCR (Dieffenbach & Dveksler 1995, McPherson & Moller 2000, Jordan *et al*. 2001).

This study aimed to investigate the prevalence of *C. gracilis* and *C. rectus* in primary endodontic infections associated with different forms of periradicular diseases using a sensitive identification method – the nested PCR.

Materials and methods

Patients and specimen collection

Adult patients ranging in age from 18–60 years who had been referred for either root canal treatment or emergency treatment to the department of Endodontics, Estácio de Sá University, Rio de Janeiro, RJ, Brazil, were selected for this study. Fifty-seven single-rooted teeth with carious lesions, necrotic pulps and radiographic evidence of periradicular disease were included. According to clinical diagnosis, cases were classified as follows: 28 cases of chronic asymptomatic periradicular lesions, 12 cases of acute apical periodontitis, and 17 cases of acute periradicular abscess. Diagnoses were based on Torabinejad & Walton (1994). None of the teeth had significant periodontal disease (pockets > 4 mm deep).

Samples were obtained under strict asepsis. The tooth was cleansed with pumice and isolated from the oral cavity with a rubber dam. Afterwards, the tooth and the surrounding field were cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite (NaOCl) solution. Complete access preparations were made using sterile burs without water spray. The operative field, including the pulp chamber, was then swabbed with 2.5% NaOCl as this solution has been demonstrated recently to be significantly more effective in decontaminating the operative field than 10% iodine prior to sampling for PCR bacterial detection (Ng *et al*. 2002). NaOCl solution was then inactivated by sterile 5% sodium thiosulphate. If upon opening the root canal was dry, a small amount of sterile saline solution was introduced into the canal. Samples were initially collected by means of a size 15 K-type file (Dentsply/Maillefer, Ballaigues, Switzerland) with the handle cut off. The file was introduced to a level approximately 1 mm short of the tooth apex, based on diagnostic radiographs, and a discrete filing motion was applied. Two sequential paper points were placed to the same level and used to soak up the fluid in the canal. Each paper point was retained in position for 1 min. The file and the two paper points were then transferred to cryotubes containing 1 mL of 5% dimethyl sulphoxide in trypticase-soy broth (TSB-DMSO; Difco, Detroit, MI, USA). Samples were immediately frozen at −20 °C.

Sampling of abscessed cases was performed after disinfection of the oral mucosa with 2% chlorhexidine.

Purulent exudate was aspirated with a sterile syringe, transferred to TSB-DMSO and frozen.

DNA extraction

Samples in TSB-DMSO were thawed to 37 °C for 10 min and vortexed for 30 s. Microbial suspension was washed three times with 100 μL of double distilled water by centrifugation for 2 min at 2500 × ***g***. Pellets were then resuspended in 100 μL of double distilled water, boiled for 10 min and chilled on ice. After centrifugation to remove cell debris for 10 s at 9000 × ***g*** at 4 °C, the supernatant was collected and used as the template for PCR amplification. Reference DNA from *C. gracilis* ATCC 33236 and from *C. rectus* ATCC 33238 was also extracted to serve as positive control for the primers used. Negative controls included the PCR mixture without DNA template or containing extracted DNA from *Actinobacillus actinomycetemcomitans* (ATCC 43718), *Bacteroides forsythus* (ATCC 43037), *C. gracilis* (for *C. rectus* primers), *C. rectus* (for *C. gracilis* primers), *C. showae* (ATCC 51146), *Capnocytophaga ochracea* (ATCC 27872), *Fusobacterium nucleatum* (ATCC 25586 and 10953), *Porphyromonas endodontalis* (ATCC 35406), *Po. gingivalis* (ATCC 33277), *Prevotella intermedia* (ATCC 25611), *Pr. nigrescens* (ATCC 33563), *Streptococcus intermedius* (ATCC 27335) and *Treponema denticola* (B1 strain, Forsyth Dental Institute).

Oligonucleotide primers

In the first PCR reaction, a practically full-length 16S rDNA was amplified using a pair of universal 16S rDNA primers, which consisted of a forward universal primer (5′-AGA GTT TGA TCC TGG CTC AG-3′, base position 8–27 relative to *Escherichia coli* 16S rDNA) and a reverse universal primer (5′-ACG GCT ACC TTG TTA CGA CTT-3′, base position 1,493–1513 relative to *E. coli* 16S rDNA).

PCR oligonucleotide primers specific for *C. gracilis* were designed using the published DNA data from the GenBank. Briefly, 16S rDNA sequences from the GenBank database were aligned, and variable areas between species were identified. Upstream and downstream *C. gracilis* primers were designed from these areas and BLAST (Altschul *et al.* 1990) was used to verify their specificity by comparing primer sequences with all available sequences in the GenBank database. BLAST search revealed no likely cross-reactivity with other related or unrelated oral species. Primer specificity was further tested against DNA from the reference strains used as controls. The PCR oligonucleotide species-specific primers, 16S rDNA-directed, for *C. gracilis* were 5′-AAC GGA ATT TAA GAG AGC TT-3′ (forward primer, located at base position 65–84 of the *C. gracilis* 16S rDNA, GenBank accession no. LO4320) and 5′-CTT TCC CGA TTT ATC TTA TG-3′ (reverse primer, located at base position 192–211 of the *C. gracilis* 16S rDNA, GenBank accession no. LO4320), producing a PCR amplicon of 147 bp.

Specific primers for *C. rectus* were 5′-TTT CGG AGC GTA AAC TCC TTT TC-3′ (forward primer, located at base position 415–437 of the *C. rectus* 16S rDNA) and 5′-TTT CTG CAA GCA GAC ACT CTT-3′ (reverse primer, located at base position 992–1012 of the *C. rectus* 16S rDNA). Predicted amplicon size is 598 bp. *Campylobacter rectus* primers were as described by Ashimoto *et al.* (1996). Primers were synthesized by Oligos Etc. Inc. (Wilsonville, OR, USA).

nPCR assay

A 16S rDNA-based nPCR detection method was used to detect *C. gracilis* and *C. rectus* in clinical samples. Aliquots of 5 μL of the supernatant from clinical samples were used as target in the first PCR reaction using universal 16S rDNA primers. PCR amplification was performed in 25 μL of reaction mixture containing 0.2 μM concentration of forward and reverse universal primers, 2.5 μL of 10× PCR buffer, 2 mM $MgCl_2$, 1.25 units of *Tth* DNA polymerase (Biotools, Madrid, Spain) and 25 μM concentration of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP and dTTP) (Gibco BRL, Gaithersburg, MD, USA).

Afterwards, 1 μL of the universal reaction was used as template for the nested specific reaction. The second PCR reaction, used to assess the occurrence of *C. gracilis* or *C. rectus*, was performed in 50 μL of reaction mixture containing 1 μM of each specific primer, 5 μL of 10× PCR buffer (Gibco BRL), 2 mM $MgCl_2$, 1.25 unit *Tth* DNA polymerase (Biotools) and 0.2 mM of each deoxyribonucleoside triphosphate (Gibco BRL). PCR reactions were performed in 25-well microtitre plates.

Preparations were amplified in a DNA thermocycler (Primus 25/96, MWG-Biotech, Ebersberg, Germany). The PCR temperature profile for the universal reaction included an initial denaturation step at 97 °C for 1 min, followed by 26 cycles of a denaturation step at 97 °C for 45 s, a primer annealing step at 55 °C for 45 s, an extension step at 72 °C for 1 min and a final step of 72 °C for 4 min. Temperature profile for the second round of amplification specific for *C. gracilis* included an initial denaturation step at 95 °C for 2 min, followed by 26 cycles of a denaturation step at 94 °C for 30 s, a primer annealing step at 53 °C for 1 min, an extension step at 72 °C

for 1 min and a final step of 72 °C for 2 min. For the detection of *C. rectus*, the second round of amplification consisted of an initial denaturation step at 95 °C for 2 min, followed by 26 cycles of a denaturation step at 95 °C for 30 s, a primer annealing step at 60 °C for 1 min, an extension step at 72 °C for 1 min, and a final extension at 72 °C for 2 min following the last cycle.

PCR amplicons were analysed by electrophoresis in a 1.5% agarose gel at 4 V cm^{-1} in Tris–borate–EDTA buffer. The gel was stained for 15 min with 0.5 μg mL^{-1} ethidium bromide and visualized under ultraviolet light. Positive reactions were determined by the presence of bands of the appropriate sizes. A 100-bp DNA ladder digest (Gibco BRL) served as the molecular size marker.

Data analysis

Prevalence values of *C. gracilis* and *C. rectus* were recorded as the percentage of the cases examined. The chi-square test was used to analyse the association between these bacterial species and the occurrence of symptoms. Significance for chi-square test was established at 1% ($P < 0.01$).

Results

Campylobacter gracilis and *C. rectus* were, respectively, detected in 21.4 (6 of 28) and 30% (6 of 20) of the root canals associated with chronic asymptomatic periradicular lesions. *Campylobacter gracilis* was found in 16.7% (2 of 12) of the cases diagnosed as acute apical periodontitis, whilst *C. rectus* was in 33.3% (two of six cases). In the abscessed cases, *C. gracilis* and *C. rectus* were detected in 23.5 (4 of 17) and 11.8% (2 of 17) of the cases, respectively. Neither *C. gracilis* ($P = 0.797$), nor *C. rectus* ($P = 0.539$) was positively associated with clinical symptoms. In general, species-specific nPCR allowed the detection of *C. gracilis* in 21.1% (12 of 57) and *C. rectus* in 23.3% (10 of 43) of the samples taken from primary endodontic infections. The species were found together in only two asymptomatic teeth.

Reference DNA and clinical samples that were positive for either *C. gracilis* or *C. rectus* showed only one band of the predicted size. Specific primers generated no amplicons with genomic DNA from nontarget bacterial species. The detection limit of the nPCR assay used in this study was approximately 10 cells as determined by amplification of serial dilutions of templates prepared from genomic DNA.

All clinical samples contained bacteria as demonstrated after the first round of amplification using universal primers for the 16S rDNA. A product of the appropriate size (1505 bp) was obtained from all samples, revealing that bacteria were present in all cases examined, demonstrating the suitability of the DNA for PCR analysis, and indicating the absence of inhibitors in the reaction mixture.

Discussion

The specificity of a microbiological diagnostic test is essential to avoid false positive results. In the present study, no evidence of cross-reactivity was observed when checking the *C. gracilis*- and *C. rectus*- specific primers against a panel of nontargeted oral species. Nonspecific amplification products were also absent. In addition, nPCR directed to 16S rDNA is more sensitive and can still show improved specificity when compared with single PCR by allowing the second species-specific reaction to be performed with reduced background of necrotic tissue, pus debris, eukaryotic DNA and other regions of the bacterial DNA.

Campylobacter gracilis have been found in infections of endodontic origin in prevalence values ranging from 1.5–55.6% of cases. Sundqvist *et al.* (1989) have recovered *C. gracilis* from 13.6% of canals containing black-pigmented rods. In another study, Sundqvist (1992) investigated the root canal microbiota of 65 teeth with intact pulp chambers and radiographic evidence of periradicular disease and found *C. gracilis* in only one case (1.5%). Gomes *et al.* (1996) isolated *C. gracilis* from 2.9% of 70 infected root canals. Le Goff *et al.* (1997) reported the highest prevalence value for this bacterial species when evaluating the microbiota of infected root canals in teeth without carious lesions and with intact crowns – 55.6% of cases. Sundqvist *et al.* (1998) have found this species in 12.5% of the canals of teeth with failed endodontic treatment. Some studies evaluating the presence of microbial species in chronic periradicular lesions have also found *C. gracilis*. Wayman *et al.* (1992) revealed that *C. gracilis* was one of the five most commonly isolated bacteria in lesions with no detectable communication with the oral cavity. Recently, *C. gracilis* was also detected in periradicular lesions of asymptomatic teeth by DNA–DNA hybridization (Sunde *et al.* 2000).

Studies have revealed that *C. rectus* may also be present in endodontic infections, in prevalence values ranging from 7.1–27.3%. Ranta *et al.* (1988) investigated the microbiota of 62 cases of periradicular lesions and found *C. rectus* in 11.3% of the cases. Sundqvist *et al.* (1989) observed the occurrence of this bacterial species in 27.3% of 22 root canals. Further, Sundqvist (1992)

has reported that *C. rectus* was isolated from 25% of 65 infected root canals. This species was positively associated with *P. endodontalis*, *Peptostreptococcus micros*, *Selenomonas sputigena*, *F. nucleatum*, *Actinomyces* sp. and *Eubacterium* sp., which may be partly dependent on the production of growth factors, such as formate, by most of these bacteria. Siqueira *et al.* (2000b) examined the microbiota of infected root canals using whole genomic DNA probes and the checkerboard DNA–DNA hybridization method and found *C. rectus* in 7.1% of the cases. Using the same method to investigate the microbiota associated with acute periradicular abscesses, Siqueira *et al.* (2001b) detected *C. rectus* in 7.4% of the abscessed cases. Other studies using the checkerboard DNA–DNA hybridization method to assess the microbiota present in periradicular lesions have reported the detection of *C. rectus* in a relatively high prevalence value (Gatti *et al.* 2000, Sunde *et al.* 2000).

Most studies using PCR methodology have either detected certain bacterial species never previously found in endodontic infections by culture (Conrads *et al.* 1997, Siqueira *et al.* 2000a, Jung *et al.* 2001, Rolph *et al.* 2001) or detected certain bacterial species usually at higher prevalence when compared with culture (Siqueira *et al.* 2001a, Hashimura *et al.* 2001). In the present study, *C. gracilis* was detected in 21.1% of the samples of all examined samples and *C. rectus* in 23.3%. These frequency rates as evaluated by highly sensitive nPCR assay were not significantly discrepant from culture studies. This suggests that the prevalence of these bacterial species has not probably been underestimated by culture and confirmed that they may be associated with endodontic infections in a reasonable number of cases. Because both bacterial species were found in asymptomatic as well as symptomatic infections in practically similar frequencies, no association with symptoms could be detected by statistical analysis.

The mechanisms of pathogenicity of *C. gracilis* are poorly understood. Its virulence factors probably include lipopolysaccharide (LPS), hydrogen sulphide and succinate. *Campylobacter gracilis* are usually less susceptible to antimicrobial agents than other oral *Campylobacter* species (Johnson *et al.* 1986, Tanner *et al.* 1992, Baron *et al.* 1993, Lee *et al.* 1993). Johnson *et al.* (1986) evaluated the *in vitro* activities of 17 antimicrobial agents against 46 clinical isolates of formate/fumarate-requiring anaerobic Gram-negative bacilli. *Campylobacter gracilis* showed some striking resistance, with penicillin being active against only 67%, the cephalosporins active against 67–89%, and clindamycin active against 67% of the strains tested. Because *C. gracilis* has been associated with serious deep-tissue infection, coupled with the relatively high frequency of antibiotic resistance, it has been considered as an important human pathogen (Johnson *et al.* 1985). Supportive evidence for this statement is still lacking.

Whereas the pathogenicity of *C. gracilis* has not been conclusively demonstrated, there is suggestive evidence that *C. rectus* is a pathogenic microorganism. *Campylobacter rectus* possesses some virulence factors that may be involved in the pathogenesis of periradicular diseases. They include an extracellular cytotoxin against polymorphonuclear neutrophils, LPS, a proteinaceous surface structure (S-layer), a native GroEL-like protein, tissue-damaging enzyme arylsulfatase and hydrogen sulphide (Gillespie *et al.* 1992, 1993, Okuda *et al.* 1997, Hinode *et al.* 1998, Zubery *et al.* 1998). The native GroEL-like protein is able to stimulate production of pro-inflammatory cytokines such as interleukin-6 and -8 (Hinode *et al.* 1998). S-layer possessing *C. rectus* cells can be resistant to complement-mediated killing and phagocytic killing by leucocytes in the absence of specific antibody (Okuda *et al.* 1997). It has been demonstrated that *C. rectus* can cause soft tissue destruction following inoculation into subcutaneous tissue of mice (Kesavalu *et al.* 1991). Live and heat-killed cells of *C. rectus* are also able to stimulate bone resorption in mice, possibly via LPS or other polysaccharide components (Zubery *et al.* 1998).

Taken together, the findings of this study indicated that *C. gracilis* and *C. rectus* participate in infections of endodontic origin. The possible involvement of these species with other human infections, including periodontal diseases and their potential virulence armamentarium might also implicate them in the pathogenesis of periradicular diseases. Nevertheless, whilst a pathogenetic role can be suspected for these species, clear evidence of causation is still lacking. Studies are necessary to elucidate the specific role played by *C. gracilis* and *C. rectus* in primary endodontic infections as well as their involvement in the pathogenesis of periradicular diseases.

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