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Oral Microbiology

A 16S rDNA-based nested PCR protocol to detect *Campylobacter gracilis* in oral infections

Protocolo de "nested" PCR baseado na fração 16S do rDNA para detecção de Campylobacter gracilis *em infecções orais*

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ABSTRACT: The aim of this study was to describe a 16S rDNA-based nested polymerase chain reaction (nPCR) assay to investigate the occurrence of *Campylobacter gracilis* in oral infections. Samples were collected from ten infected root canals, ten cases of acute periradicular abscesses and eight cases of adult marginal periodontitis. DNA extracted from the samples was initially amplified using universal 16S rDNA primers. A second round of amplification used the first PCR products to detect *C. gracilis* using oligonucleotide primers designed from species-specific 16S rDNA signature sequences. The nPCR assay used in this study showed a detection limit of 10 *C. gracilis* cells and no cross-reactivity was observed with nontarget bacteria. *C. gracilis* was detected in the three types of oral infections investigated – 4/10 infected root canals; 2/10 acute periradicular abscesses; and 1/8 subgingival specimens from adult periodontitis. The method proposed in this study showed both high sensitivity and high specificity to directly detect *C. gracilis* in samples from root canal infections, abscesses, and subgingival plaque. Our findings confirmed that *C. gracilis* may be a member of the microbiota associated with distinct oral infections, and its specific role in such diseases requires further clarification.
DESCRIPTORS: *Campylobacter; Campylobacter* infections; Polymerase chain reaction.

RESUMO: O objetivo deste estudo foi descrever um método de "nested" PCR baseado na fração 16S do rDNA para investigar a ocorrência de *Campylobacter gracilis* em infecções orais. Amostras foram coletadas de dez casos de canais radiculares infectados, dez casos de abscesso perirradicular agudo e oito casos de periodontite do adulto. O DNA extraído das amostras foi inicialmente amplificado usando "primers" universais para o gene do 16S rDNA. Uma segunda etapa de amplificação empregou os produtos de PCR gerados na primeira reação para detectar *C. gracilis* usando "primers" desenhados a partir de uma região específica para essa espécie localizada no gene do 16S rDNA. O método usado neste estudo apresentou um limite de detecção de 10 células de *C. gracilis* e ausência de reatividade cruzada com outras espécies bacterianas orais. *C. gracilis* foi detectado nos três tipos de infecções orais investigadas – 4/10 canais radiculares infectados; 2/10 casos de abscesso perirradicular agudo; e 1/8 espécimes subgengivais de casos de periodontite do adulto. O método proposto neste estudo foi altamente sensível e específico na detecção direta de *C. gracilis* em amostras clínicas de infecções endodônticas, abscessos e placa subgengival. Nossos achados confirmam que *C. gracilis* pode ser um membro da microbiota associada com infecções orais distintas e seu papel específico em tais doenças requer posterior elucidação.
DESCRITORES: *Campylobacter;* Infecções por *Campylobacter*; Reação em cadeia da polimerase.

INTRODUCTION

Campylobacter gracilis is a non-motile, non-sporeforming, anaerobic Gram-negative rod with a formate- and fumarate-requiring metabolism. Many strains can even be microaerophiles[3]. Formate is oxidized to produce hydrogen and carbon dioxide, while fumarate is reduced to succinate. The specific epithet of *C. gracilis* was due to the slender tapered shape of cells after 4 to 6 days of anaerobic incubation on agar plates. Cells are small and straight, 0.4 µm wide by 4 to 6 µm long, with round ends. The species has a G+C content of 43 to 47 mol%[14].

This species was proposed and described by Tanner *et al.*[14], in 1981, as *Bacteroides gracilis*. Further analyses of cellular fatty acid profiles indicated that *B. gracilis* were not "true" bacteroides[2]. In 1995, Vandamme *et al.*[15], based on an analysis of the cellular fatty acids, respiratory quinones, and proteins of *B. gracilis*, and using previously published data for genotypic and phenotypic characteristics of this species, transferred it to the genus *Campylobacter*.

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C. gracilis has been considered a probable important medical pathogen. Its primary habitat appears to be the gingival crevice and the majority of the infections caused by *C. gracilis* occur in the head and neck areas. It has been found associated with cases of root canal infections, gingivitis, adult periodontitis and periimplantitis[5,6,11-13]. Infections in the pleuropulmonary system and in the abdominal cavity as well as tubo-ovarian abscesses caused by *C. gracilis* have also been reported[4,7,16]. The potential difficulties in treating *C. gracilis* infections have been considered a major clinical concern in the management of some of these diseases[4].

Because this species is not always easily identified by conventional culture methods, it is possible that its prevalence has been underestimated in some oral infections. Molecular methods, 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 to cultivate, unculturable species, and strains within a species that show a phenotypically divergent behavior and thereby are difficult to be identified by culture procedures. The PCR methodology has the highest detection rate between the microbiological identification methods, and under optimized conditions also shows high specificity. The nested PCR (nPCR) technique is a modification of the PCR technology that involves a first round of amplification of a DNA sequence with one set of primers followed by a second round of amplification using a second set of primers complementary to smaller sequences within the first PCR product. nPCR possesses increased detection rate and specificity when compared with single PCR.

The purpose of this study was to describe a species-specific nPCR assay to directly survey clinical samples from three types of oral infections for the presence of *C. gracilis*.

MATERIALS AND METHODS

Subjects and sampling procedures

The material used in this study consisted of samples collected from adult patients with ages ranging from 18 to 65 years who had been referred for endodontic, emergency, or periodontal treatment to the School of Dentistry, Estácio de Sá University, Rio de Janeiro, RJ, Brazil. Samples were obtained from 10 infected root canals, 10 cases of acute periradicular abscesses, and 8 cases of adult marginal periodontitis. Cases of infected root canals and periradicular abscesses showed carious lesions, necrotic pulps and radiographically detected periradicular bone destruction. Periodontitis subjects had at least eight sites with pocket depths > 4 mm and no prior periodontal therapy.

Samples from infected root canals were collected as follows: after each tooth was cleansed with pumice and isolated with a rubber dam, the tooth and the surrounding field were then cleansed with 3% hydrogen peroxide and decontaminated with a 2.5% sodium hypochlorite 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% sodium hypochlorite. NaOCl solution was then inactivated by sterile 5% sodium thiosulphate. If 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 #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. Afterwards, two sequential paper points were placed up 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 cut file and the two paper points were then transferred to cryotubes containing 1 ml of 5% dimethyl sulfoxide in trypticase-soy broth (TSB-DMSO) (Difco, Detroit, MI, USA) .

Samples of acute periradicular abscesses were collected as follows: after disinfection of the oral mucosa with 2% chlorhexidine, purulent exudate was aspirated using a sterile syringe. Pus was then transferred to TSB-DMSO and frozen.

Samples of adult marginal periodontitis were collected from 8 patients as follows: after sample sites were isolated with cotton rolls and air dried, the supragingival plaque was removed and paper points were inserted up to the depth of the pockets. Samples were taken from the 3 deepest periodontal pockets in each patient. Paper points were removed after 10 seconds and then placed into cryotubes containing TSB-DMSO and frozen. Thus, periodontal samples from each patient consisted of a pool of the three sampled diseased sites.

After collection, all samples were immediately frozen at –20°C.

Sample processing for PCR

The samples in TSB-DMSO were thawed at 37ºC for 10 min and vortexed for 30 s. Microbial suspension was washed 3 times with 100 µl of bidistilled water by centrifugation for 2 min at 2,500 x g. Pellets were then resuspended in 100 µl of bidistilled water, boiled for 10 min and chilled on ice. After centrifugation to remove cell debris for 10 s at 9,000 x g at 4ºC, the supernatant was collected and used as the template for PCR amplification. Reference DNA from *C. gracilis* ATCC 33236 was also extracted to serve as positive control for the primers used. Extracted DNA from *Actinobacillus actinomycetemcomitans* (ATCC 43718), *Bacteroides forsythus* (ATCC 43037), *Campylobacter rectus* (ATCC 33238), *Campylobacter showae* (ATCC 51146), *Capnocytophaga ochracea* (ATCC 27872), *Fusobacterium nucleatum* (ATCC 25586 and 10953), *Porphyromonas endodontalis* (ATCC 35406), *Porphyromonas gingivalis* (ATCC 33277), *Prevotella intermedia* (ATCC 25611), *Prevotella nigrescens* (ATCC 33563), *Streptococcus intermedius* (ATCC 27335), and *Treponema denticola* (B1 strain, Forsyth Dental Institute, Boston, MA, USA) was also used to check for the specificity of *C. gracilis* primers.

Universal 16S rDNA 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 1,493–1,513 relative to *E. coli* 16S rDNA).

Design of *C. gracilis* specific primers

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[1] 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 acession no. L04320) and 5'- CTT TCC CGA TTT ATC TTA TG -3' (reverse primer, located at base position 211-192 of the *C. gracilis* 16S rDNA, GenBank acession no. L04320), producing a PCR amplicon of 147 bp. 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* in clinical samples. Aliquots of 5 µl of the supernatant from clinical samples were used as target in the first round of PCR amplification 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 X PCR buffer, 2 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md, USA) and 25 µM concentration of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP) (Gibco BRL).

Afterwards, 1 µl of the universal reaction was then used as template for the second round of PCR amplification, which was directed towards the specific detection *C. gracilis*. The second PCR reaction was performed in a 50 µl of reaction mixture containing 1 µM of each specific primer, 5 µl of 10 X PCR buffer (Gibco BRL), 2 mM $MgCl_2$, 1.25 unit *Taq* DNA polymerase (Gibco BRL) and 0.2 mM of each deoxyribonucleoside triphosphate (Gibco BRL). PCR reactions were performed in 25-well microtiter plates.

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

PCR products were analyzed by electrophoresis in a 1.5% agarose gel at 4 V/cm in Tris-borate-EDTA buffer. The gel was stained for 15 min with 0.5 µg/ml ethidium bromide and photographed 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 standard.

RESULTS

Amplification of reference genomic DNA of *C. gracilis* ATCC 33236 yielded a single 147 bp band. Clinical samples that were positive for *C. gracilis* also showed only one band of the predicted size. The detection limit of the nPCR assay used in this study was approximately 10 *C. gracilis* cells as determined by amplification of serial dilutions of templates prepared from *C. gracilis* genomic DNA. No amplification was detected for any of the reference strains that were not *C. gracilis*. Figure 1 depicts representative *C. gracilis* specific amplicons obtained after nPCR amplification of reference DNA and clinical samples.

Species-specific nPCR for *C. gracilis* allowed the detection of this species in 4/10 infected root canals, 2/10 abscessed cases, and 1/8 subgingival specimens of adult marginal periodontitis.

The first round of amplification using universal primers for the 16S rDNA generated a product of the expected size (1,505 bp). This revealed that bacteria were present in all cases examined, demonstrated the suitability of bacterial DNA for PCR analysis, and indicated that PCR was conducted without significant inhibition by components of the clinical samples.

DISCUSSION

The nPCR assay described in this study to directly detect *C. gracilis* in oral infections showed both high sensitivity and high specificity. The specificity of the oligonucleotide primers is important to avoid false positive results when using PCR. *C. gracilis* is closely related to *C. rectus* and *C. showae*, based on 16S rDNA sequencing, and shares many of their characters. We observed no evidence of cross-reactivity when checking the *C. gracilis* specific primers against a panel of twelve non-targeted oral species, including the closely related species *C. rectus* and *C. showae*. Non-specific amplification products were also absent. Therefore, the method's specificity for *C. gracilis* detection was high.

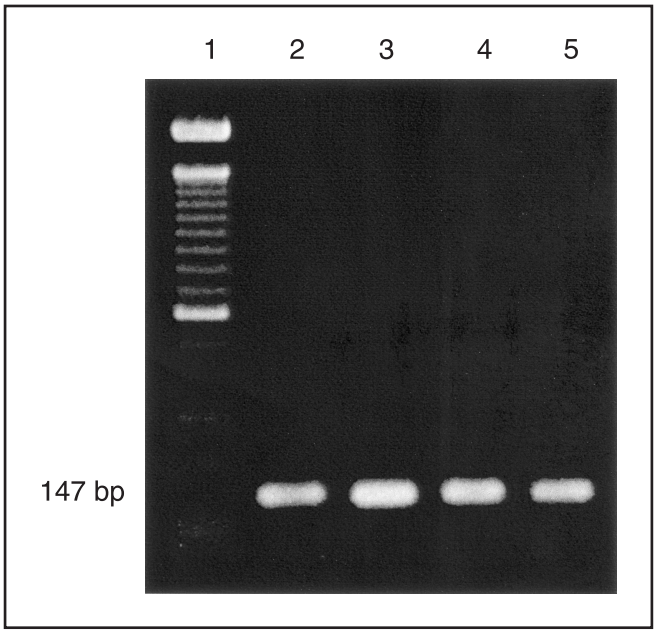

FIGURE 1 - Representative PCR generated amplicons in electrophoresis. Lane 1, 100 bp ladder; Lanes 2 to 5, amplification using *Campylobacter gracilis* specific primer; Lane 2, *C. gracilis* DNA (control); Lanes 3 to 5, *C. gracilis* in a clinical sample.

The high detection rate of nPCR approaches may be a reason of concern, particularly when non-quantitative assays are employed. It has been claimed that because PCR can detect a very low number of cells of a given species, the results obtained by this method may not have significance with regard to etiology of a given disease. Although this must be really taken into account when analyzing nPCR results, some other factors should be also considered in this discussion, which represent great advantages of such highly sensitive identification procedure. First, because there is no agreed bacterial load for clinically inducing periradicular and periodontal diseases, it would be obviously prudent that the method of assay should detect the smallest number possible of bacterial cells in samples. Here lies one of the greatest advantages of the nPCR. Second, the method used in this study has a detection limit of approximately 10 cells of the target species. If one considers that a 5% volume of the original sample (100 µl) was used in individual PCR experiments, the detection limit was then approximately 200 cells in the whole sample, which is still more sensitive than other

identification methods but can be also significant with regard to etiology of the disease.

C. gracilis was detected in the three types of oral infections examined. Prevalence values do not deserve speculations about the role of this species in each disease because of the low number of samples investigated. However, our findings demonstrated that the method was effective in detecting *C. gracilis* in samples from root canal infections, abscesses, and subgingival plaque. All these infectious diseases are usually characterized by a mixed infection[8-11]. Therefore, the nPCR method used herein also showed the capacity to detect *C. gracilis* against a background of other bacterial species.

CONCLUSION

The 16S rDNA-based nPCR approach proposed in this study to identify *C. gracilis* was highly sensitive, specific, and capable to directly detect this species in clinical samples from oral infections. The use of this method may help to delineate the role of *C. gracilis* in different oral infectious diseases.

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