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Molecular Identification and Phylogeny of Commonly Occurring Periodontal Bacteria using 16S rRNA Gene Sequences

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This study reports the detection and identification of four commonly occurring dental bacteria including *Prophyromonas gingivalis*, *Bacteroides forsythes*, *Actinobacillus actinomycetenicomitans* and *Prevotella intermedia* using PCR amplification with 16S rRNA sequence-specific primers. The phylogenetic relationship among these bacteria was also studied after retrieving 16S rRNA gene sequences from the GenBank nucleotide database. There were positive amplifications for *Actinobacillus actinomycetenicomitans* in 6 out of 8 samples followed by *Prophyromonas gingivalis* (5/8) and *Bacteroides forsythes* (3/8) whereas only one sample was positive for *Prevotella intermedia*. Multiple sequence alignment showed a large number of variable sites (547 out of post-alignment 1510 sites) while the average frequencies of conserved sequences among the species were 60.9%. The phylogenetic tree clearly differentiated *Actinobacillus actinomycetenicomitans* and *Prevotella intermedia* from *Prophyromonas gingivalis* and *Bacteroides forsythes*, the later two species belong to the same family (but different genera) and appeared as a separate clade. The hierarchical flow of the tree clearly showed the discriminatory power of 16S rRNA marker from phylum to genus level. In conclusion, PCR amplification using 16S rRNA sequence-specific primers provides a sensitive and specific method for identification of dental bacteria. This method does not require any culturing or DNA extraction hence is more simple and time saving. Post-amplification sequencing of 16S rRNA provides a reliable tool for phylogenetic and molecular diversity analyses.

Key Words: Dental bacteria; Molecular identification; PCR; Phylogenetic analysis; 16S rRNA.

Dental caries and gingivitis are the most prevalent oral infectious diseases of humans and are due to the accumulation of a microbial biofilm (dental plaque) on the tooth surface and at the gingival margin, respectively[1]. Periodontal disease is highly prevalent in diabetic patients as compared to healthy subjects[2]. Increasing age and smoking have been found to increase the likelihood of severe periodontal disease in diabetic patients[3]. Poor oral health may adversely affect diabetic glycemic control and subsequent medical complications[4]. Even the diabetic status is significantly associated with attachment and bone loss[4]. The initiation and progression of periodontitis is caused by several microbiota

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accumulated in the subgingival pockets. Subgingival flora are complex and may contain over 250 species, however, only few of them including *Prophyromonas gingivalis*, *Bacteroides forsythes*, *Actinobacillus actinomycetenicomitans* and *Prevotella intermedia* have been described as periodontopathic bacteria[5].

Many of the clinical laboratories rely on manual, automated, or semiautomated phenotypic methods and commercial systems for identification of bacterial pathogens. Conventional phenotypic identification may involve a number of methods, including observation of growth and colony morphology on various media, analysis of manual biochemical reactions, and the use of automated and nonautomated commercially available biochemical panels. Unfortunately, the commercial phenotypic databases are often outdated and lack current taxonomy[6]. Moreover, phenotypic systems often cannot account for the variable characteristics observed among members of the same species, resulting in poor precision[7]. It has been suggested that conventional biochemical or Gram reactions are not foolproof and may lead to inappropriate use of comparative databases while such exhaustive phenotypic testing potentially delays turnaround time without the added benefit of accuracy[8].

Genotypic identification of microorganisms by 16S rRNA gene sequencing has emerged as a more objective, accurate, and reliable method for bacterial identification, with the added capability of defining taxonomical relationships among bacteria[9]. 16S rRNA gene sequencing is particularly important for bacteria with unusual phenotypic profiles, rare bacteria, slow growing bacteria, uncultivable bacteria and culture-negative infections. The oral cavity or dental-related specimens and the gastrointestinal tract are the most important sites for discovery or reservoirs of novel species[10]. About half of the oral microflora is unculturable thus targeted culture-independent molecular ecology studies could play a valuable role in the identification of bacterial targets for further investigations of the pathogenesis of bacterial infections[11].

16S rRNA gene sequence analysis is widely preferred for species delineation for two important reasons: 16S rRNA gene sequence identity greater than 97% may indicate a specific species and sequencing the 16S rRNA gene has become much cheaper and faster due to technological advancements. Microbial diversity within the human body has been quantified through 16S rRNA surveys[12]. Such 16S rRNA surveys are being increasingly applied in clinical settings to determine how microbiota correlate with human disease states[13]. Phylogenetic analysis plays an important role in characterization of microflora for clinical implications[14]. 16S rRNA gene has been regarded as a suitable microbial phylogenetic marker[15]. Although molecular methods of bacterial identification have been used for different pathogens, fewer studies have used them for identification of oral microflora[16].

This investigation was aimed to study the phylogenetic relationship among four commonly occurring dental bacteria including *Prophyromonas gingivalis*, *Bacteroides forsythes*, *Actinobacillus actinomycetenicomitans* and *Prevotella intermedia* using 16S rRNA gene sequences. The conventional taxonomic relationship of these bacteria is shown in Table 1. *Actinobacillus actinomycetenicomitans* differs from the other three bacteria at phylum level whereas *Prevotella intermedia* differs from the remaining two bacterial species at family level. *Prophyromonas gingivalis* and *Bacteroides forsythes* are closely related and represent the divergence at the genus level (Table 1).

MATERIALS AND METHODS

Identification of dental bacteria by PCR

The subgingival plaque samples were collected from four sites (about 5 mm pocket depth) per individual using separate sterile curettes and a single vertical stroke. Each sample was immediately placed in a sterile Eppendorf tube containing 0.5 ml Tris-EDTA buffer (10 mM Tris HCl (pH 7.6), 1 mM EDTA (pH 8.0). For PCR analysis, 90 µl of vortex-mixed subgingival plaque sample was added to 10 µl of 10 x lysis buffer (100 mM Tris-HCl, pH 8.0 + 10 mM EDTA + 10% Triton X-100) and boiled for 5 min; 5 µl of this lysate was used in each PCR reaction. The primer pairs used for the various PCR analyses were as follows: *Prophyromonas gingivalis* (5-TGT AGA TGA CTG ATG GTG AAA ACC; 5-ACG TCA TCC CCA CCT TCC TC), *Bacteroides forsythes* (5-GCG TAT GTA ACC TGC

CCG CA; 5-TGC TTC AGT GTC AGT TAT ACC T), *Actinobacillus actinomycetenicomitans* (5-AGA GTT TGA TCC TGG CTG AG; 5-CAC TTA AAG GTC CGC CTA CGT GCC) and *Prevotella intermedia* (5-TTT GTT GGG GAG TAA AGC GGG; 5-TAC ACA TCT CTG TAT CCT GCG T)

PCR amplification was carried out in a reaction volume of 25 µl consisting of 5 µl sample lysate and 20 µl of reaction mixture containing 1x PCR buffer (10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 2 units of Taq DNA polymerase, 0.2 mM dNTPs and 100 pmol of each primer. The cycling conditions were initial denaturation for 5 min at 95°C, 35 amplification cycles of denaturation at 95°C for 1 min, annealing of primers at 60°C for 1 min and primer extension at 72°C for 1.5 min, followed by a final extension step at 72°C for 7 min. The cycling conditions were same for all the bacterial species except for *Prevotella intermedia* the annealing temperature was 58°C. After PCR amplification, 10 µl of each reaction product was fractionated on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. Gel images were obtained using Proxima C16 Phi+ (Isogen Life Science) UV transilluminator and Opticom (version 3.2.5; OptiGo) imaging system.

Table 1. Taxonomic relationship of dental bacteria used in this study.

Kingdom	Bacteria	Bacteria	Bacteria	Bacteria
Phylum	Bacteroidetes	Bacteroidetes	Bacteroidetes	Proteobacteria
Class	Bacteroidia	Bacteroidia	Bacteroidia	Gammaproteobacteria
Order	Bacteroidales	Bacteroidales	Bacteroidales	Pasteurellales
Family	Porphyromonadaceae	Porphyromonadaceae	Prevotellaceae	Pasteurellaceae
Genus	Porphyromonas	Bacteroides	Prevotella	Actinobacillus
Species	Gingivalis	Forsythus	Intermedia	Actinomycetemcomitans

Table 2. Pair-wise sequence comparisons showing the actual number of variable sites (upper right) and the number of base substitutions per site (lower left) using the maximum composite likelihood model.

	P.gingivalis	*B.forsythus*	*P.intermedia*	*A.actinomycetemcomitans*
P.gingivalis		154	265	385
B.forsythus	0.1194		242	373
P.intermedia	0.2197	0.1972		399
A.actinomycetemcomitans	0.3474	0.3318	0.3626	

Phylogenetic analysis of dental bacteria

The 16S rRNA gene sequences of *Prophyromonas gingivalis*, *Bacteroides forsythes*, *Prevotella intermedia* and *Actinobacillus actinomycetenicomitans* were obtained from the GenBank nucleotide database. The respective GenBank accession numbers are as follows: L16492, L16495, L16468 and M75037. The sequences were aligned by ClustalW[17] and then subjected to maximum parsimony (MP), neighbor joining (NJ) and unweighted pair group method with arithmetic mean (UPGMA) for phylogenetic analyses using the evolutionary distances computed by maximum composite likelihood method. All these analyses were performed using MEGA4 software and the bootstrap consensus trees inferred from 1000 replicates were taken to represent the evolutionary history of the taxa analyzed[18,19].

RESULTS

PCR-based identification of dental bacteria

PCR amplification using sequence-specific primers for *Prophyromonas gingivalis*, *Bacteroides forsythes*, *Actinobacillus actinomycetenicomitans* and *Prevotella intermedia* resulted intense gel bands of anticipated sizes, 197, 641, 593 and 575 bp, respectively (Fig. 1). There were positive amplifications for *Actinobacillus actinomycetenicomitans* in 6 out of 8 samples followed by *Prophyromonas gingivalis* (5/8) and *Bacteroides forsythes* (3/8) whereas *Prevotella intermedia* was detected in only one sample (Fig. 1).

In-silico phylogeny of dental bacteria

The total numbers of nucleotides for the 4 bacterial species were as follows: *Prophyromonas gingivalis*, 1467; *Bacteroides forsythes*, 1455; *Prevotella intermedia*, 1460; and *Actinobacillus actinomycetenicomitans*, 1478 while the numbers of conserved (identical) sites for these species were found to be 925, 922, 919 and 911 respectively. The average frequencies of conserved sequences among the species were 60.9%. The topologies of all the trees were identical irrespective of the method applied such as MP, NJ or UPGMA (Fig. 2). This is due to a large number of variable sites (547 out of post-alignment 1510 sites) and the gross differences among bacterial species at various

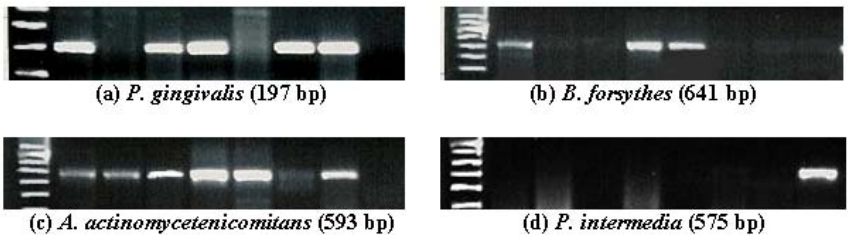

Fig. 1. Agarose gel bands showing the identification of periodontal bacteria in dental plaque samples by PCR. The extreme left lane is a size marker. Abbreviation: bp= base pair.

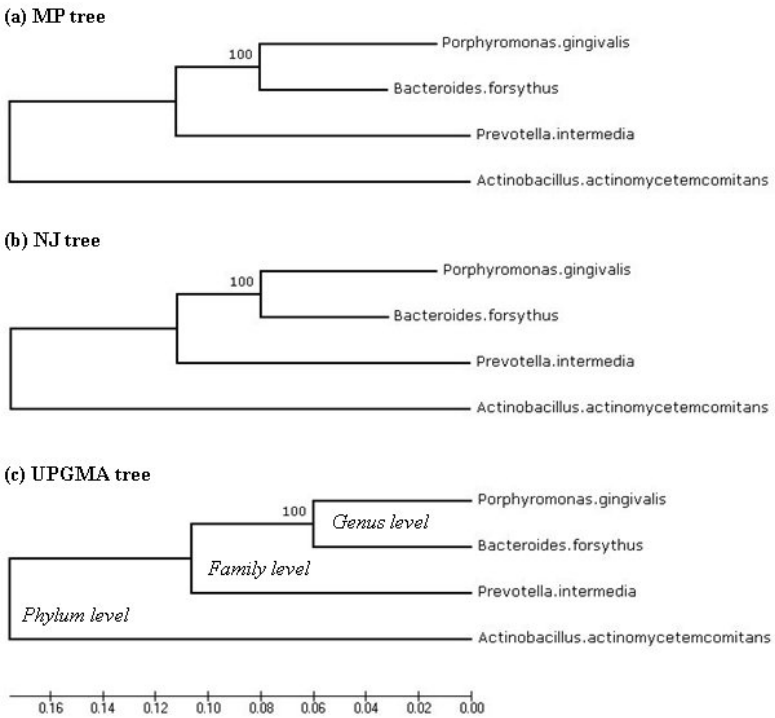

Fig. 2. MP, NJ and UPGMA trees showing the relationship among the four different species of dental bacteria

taxonomic levels. The phylogenetic tree clearly differentiated *Actinobacillus actinomycete-nicomitans* and *Prevotella intermedia* from *Prophyromonas gingivalis* and *Bacteroides forsythes*, the later two species belong to the same family (but different genera) and appeared as a separate clade. The hierarchical flow of the tree clearly showed the discriminatory power of 16S rRNA marker from phylum to genus level (Fig. 2).

DISCUSSION

The results of this study showed that PCR amplification using 16S rRNA sequence-specific primers provides a sensitive and specific method for identification of dental bacteria (Fig. 1). Since this method does not require any culturing or DNA extraction it is more simple and time saving. 16S rRNA genotyping not only provides insights into etiologies of infectious disease but it also helps clinicians in choosing antibiotics and in determining the duration of treatment and infection control procedures[10]. Petti *et al*[8] have identified three cases of endocarditis where the initial identification by phenotypic methods was erroneous and genotypic identification by 16S rRNA gene sequencing and phylogenetic analysis provided clinicians with a more accurate and meaningful result. Amplification using 16S rDNA primers has been proposed as a strategy for the diagnosis of culture-negative bacterial meningitis and applied in daily microbiological practice[20]. PCR-based identification of bacteria is extremely useful when an early intervention of disease is crucially important, such as sepsis[21]. Springer *et al*[22] have shown that molecular typing by 16S rRNA sequence determination is not only more rapid (12 to 36 h versus 4 to 8 weeks) but also more accurate than traditional typing and therefore is a standard means for identification of microorganisms in the clinical laboratory, especially microorganisms which are difficult to identify by the use of more traditional techniques.

16S rRNA gene sequencing is a more objective identification tool, unaffected by phenotypic variation or technologist bias, and has the potential to reduce laboratory errors[8]. Sequencing of 16S rRNA gene identified 88 isolates (91.7%) with >99% similarity to a sequence from the assigned species; 61.5% of sequencing results were concordant with phenotypic results, indicating the usability of sequencing to identify Gram-negative bacilli[23]. Because 16S rRNA gene sequence analysis can discriminate far more finely among strains of bacteria than is possible with phenotypic methods, it can allow a more precise identification of poorly described, rarely isolated, or phenotypically aberrant strains[9]. This is an area in which 16S rRNA gene sequence identification might have an immediate impact on patient care. For example, some species of "viridans" streptococci are far more likely than others to cause endocarditis; identification of these organisms by phenotypic methods is difficult and erroneous. Thus, judging the importance of isolates of "viridans" streptococci isolated from the blood has been a problem for the infectious-disease physician however 16S rRNA gene sequence analysis provides accurate identification at the species level and can clarify their clinical importance[24].

The results of phylogenetic analysis clearly discriminated the four bacterial species with high bootstrap support (Fig. 2). Post amplification sequencing of 16S rRNA gene is known to facilitate phylogenetic analysis and differentiation of bacteria at the species or strain level[25]. Molecular studies of microbial diversity have provided many insights into the bacterial communities inhabiting the human body while a common primary step is a survey of conserved marker genes, particularly 16S rRNA, to characterize the taxonomic composition and diversity of these communities[26]. McLaughlin *et al*[27] have reported 16S rRNA based identification and phylogenetic analysis of analhmicrobiota in patients with and without pouchitis after restorative proctocolectomy (RPC) for ulcerative colitis and familial adenomatous polyposis. Hong *et al*[28] have used 16S rRNA-based pyrosequencing to provide a comparative analysis of fecal microbial diversity in land and marine iguanas. A recent meta analysis of ruminal microorganisms diversity showed that 16S rDNA sequence database entries assigned to 5271 operation taxonomic units (OTUs) at species level (0.03 phylogenetic distance) and more than 3500 OTUs at genus level (0.05 distance)[29]. OTUs are conventionally defined at a phylogenetic distance (0.03-species, 0.05-genus, 0.10-family) based on full-length 16S rRNA gene sequences. Thus, the phylogeny of dental bacteria using 16S rRNA gene allows species level discrimination.

However, the approach may have some limitations, particularly in discriminating very closely related taxa[30].

In conclusion, PCR-based amplification of 16S rRNA gene using sequence-specific primers offers a simple and robust method for molecular identification of dental bacteria. Moreover, 16S rRNA gene sequence information provides a powerful tool for the phylogenetic inference of dental bacteria that may have relevant application in clinical pathologies.

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