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16S rDNA sequence analysis of periodontitis microbiota: A Pilot Study

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Introduction
Pathogenic bacterial colonization/re-colonization of subgingival niches is the main cause of periododontitis/recurrent periodontitis. Certain predominately cultivable periodontopathogens e.g. Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis out of the total approx. 300 cultivable oral microbes were shown to be implicated in the disease (Darveau et al. 1997). These incriminated microbes, however, were repeatedly reported to comprise only a minor proportion of the subgingival microflora (Lyons et al. 2000, Rudney et al. 2003). New uncultured periodontopathogens have been recently identified (Sakamoto et al. 2002, Brinig et al. 2003). There is still uncertainty regarding what pathogens or combinations of pathogens play a major role in periodontal infection. Current research reports had indicated that potentially 10-50% of the subgingival flora is unidentified and hence, by inference, up to 50% of the periodontopathogens may yet be unknown to the scientific world (Paster et al. 2001, Hutter et al. 2003). Traditional culture dependent bacterial isolation and characterization, is not error proof. Biochemical or abbreviated molecular identification provides the best possible identification limited only by current scientific knowledge. For accurate study of periodontitis microbiota, more specific approaches are required (Tanner et al. 1994). 16S ribosomal DNA sequences are one current tool for phylogeny of microbes (Hugenholtz et al. 1998) and this approach is the current “gold standard”. Kroes et al. (1999), Paster et al. (2001) and Hutter et al. (2003) had demonstrated, utilizing 16S rDNA gene analysis technique, great diversity of the subgingival microflora associated with periodontal health and disease conditions. Initial data from a variety of clinical situations are available from the US and Europe (Kroes et al. 1999, Spratt et al. 2001, 2002, Kazor et al. 2003, Hutter et al. 2003). Many novel 16S rDNA sequences could be identified from the above studies, however, their relevance to periodontitis is yet to be discovered.

Aim
Our aim is to study the microbial diversity of periodontitis subgingival microbiota by cloning and partial sequencing of 16S rDNA gene.

Material & Methods

Plaque Sample
From diseased site of untreated periodontitis patient

DNA Extraction
Lysozyme
Trex-4HC (pH 7.6), EDTA (pH 8), Tween 20 & Proteinase K
Wash with PBS

Gene Amplification
16S rDNA gene were amplified with a universal primer set: Forward primer: 5’-GAGAAGTTTGTGATCCTTGAGCG-3’
Reverse primer: 5’-GGAAGGACTTACACAGCGAAG-3’
PCR was performed in GeneAmp® PCR System 9700 thermal cycle (Applied Biosystems)

Gel Electrophoresis
PCR product with 1,500bp was obtained

PCR Purification
QIAquick® Gel Extraction kits (Qiagen)

Transformation
TA Cloning kit (Invitrogen)
Vector: pCR 2.1 TOPO TA
According to the instructions of the manufacturer

Colonoy Selection
Number of Colony Selected: 120
Number of Colony with correct clone: 86

DNA Sequencing
Primer: M13 (-20) reverse primer
Sequencing reactions were performed in ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems)

BLAST
Nucleotide-nucleotide BLAST (NCBI)

Phylogenetic Analysis
Phylogenetic tree was constructed using neighbor joining method (PHYML v3.1)

Results

| 408 – 1546 bases, mean 645 ± 221 bases sequenced from the 86 correct transformants |
| total of 35 clones identifiable |
| 7 clones (44.2%, mean 1016 ± 501 bases sequenced) were identified as known characterized bacterial species |
| 12 clones (20.9%, mean 804 ± 426 bases sequenced) were identified as partially characterized bacterial species/strains |
| 14 clones (16.3%) were of ≤ 97% similarity with NCBI database |
| A total of 16 clones (34.9%, mean 582 ± 154 bases sequenced) belonged to uncharacterized bacterial strains |

Conclusions

• Within the limitations of the culture independent 16S rDNA cloning and sequencing method, we found that about one half of the studied periodontitis subgingival microbiota comprised of partially- or uncharacterized microorganisms.

• More research efforts should be directed to culture independent study of periodontitis associated subgingival microbial biofilms.

Acknowledgement
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Reference
Hutter, G. et al. (2003) Microbiology 149:67
Spratt, D. A. et al. (1999) Oral Microbiology and Immunology 14:56