American Journal of Biochemistry and Biotechnology 4 (4): 367-370, 2008
ISSN 1553-3468

Identification of Major Cultivable Aerobic Bacteria in the Oral Cavity of Malaysian Subjects

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Abstract: Culture dependent and culture independent methods have shown that about 600 species of bacteria inhabit the human oral cavity. While some oral microorganisms have a direct link to dental caries, periodontal disease and halitosis, opportunistic pathogens may be responsible for systemic diseases such as bacterial endocarditis, aspiration pneumonia, osteomyelitis in children, preterm low birth weight, coronary heart disease and cerebral infarction (or stroke). This study employs bacterial 16S rDNA sequences to rapidly identify the major cultivable aerobic bacteria in the oral cavity of Malaysian subjects. The data obtained shows that the oral cavity of healthy volunteers contains a number of potentially pathogenic organisms including *Streptococcus pneumoniae* and *Staphylococcus aureus.* The need to profile and characterize these microorganisms using rapid detection methods can go a long way in developing future management strategies in clinical setting to enhance oral health in the Malaysian population.

Key Words: 16S rDNA sequences, oral cavity, pathogenic

INTRODUCTION

Culture-dependent and culture-independent methods have estimated that about 600 species of bacteria inhabit the human oral cavity. The oral microbiotas play critical roles in oral health and are directly linked to diseases such as dental caries, periodontal disease and halitosis. *Streptococcal* species (*S. mutans, S. sobrinus*), *Lactobacilli*, *Actinomyces* and occasionally *Candida* yeasts have been implicated in dental caries[8]. *Porphyromonas*, *Bacteroides*, *Prevotella* species have been associated with peridontal disease and halitosis[9,10].

The oral cavity is also inhabited by many types of lactic acid bacteria that are able to inhibit oral pathogens by producing hydrogen peroxide, bacteriocins and organic acids. Such bacteria include *Lactobacillus rhamnosous GG*, *Lactobacillus casei*, *Bifidobacterium*, *Streptococcus oligofermentas*, *Streptococcus mutans, Weissella cibaria* and *Streptococcus salivarius*[1,2,4,12].

The oral cavity may serve as a reservoir for many pathogenic bacteria. Opportunistic oral bacteria have been documented in causing systemic diseases such as bacterial endocarditis, aspiration pneumonia, osteomyelitis in children, preterm low birth weight, coronary heart disease and cerebral infarction or stroke[11].

The development of the oral community involves competition as well as synergy among these bacteria. The bacterial populations in the human oral cavity are in a dynamic state of change. The need to profile and characterize these microorganisms using an appropriate rapid identification method can go a long way in enhancing oral health management in the Malaysian population. The aim of this study was to identify the major cultivable aerobic microbial population of the oral cavity by using 16S rRNA gene sequencing analysis.

MATERIALS AND METHODS

Sampling sites for bacteria were the dorsum of the tongue, teeth surface and gingival crevice of healthy subjects. Bacteria were collected using either the Gracey curette (teeth surface and gingival crevice) or cotton swab (tongue). Swabbed samples were suspended in Reduced Transport Fluid (RTF) and

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diluted 10, 10^2 and 10^3 times. Diluted samples were plated in duplicate on Columbia blood agar plates and incubated aerobically at 37°C for 3-5 days. Distinct colonies (2-3 colonies of apparently same morphology) were selected and streaked separately on agar plates. Single colonies obtained were characterized by gross (colour and shape) and microscopic morphology as well as by gram staining. The genomic DNA of pure cultures was prepared using i-genomic DNA extraction mini kit (iNtRON Biotechnology, Seongnam). Extracted DNA was evaluated for both quality and quantity by agarose gel (1%) electrophoresis. Universal primers for 16S rDNA (F: 5'-AGA GTT TGA TCA TGG CTC AG and R: 5'-TAC GGC TAC CTT GTT ACG ACTT) were used for PCR amplification. PCR conditions were: initial denaturation at 94°C for 5 min., denaturation at 94°C for 1 min., annealing at 52°C for 1 min., extension at 72°C for 1.5 min and final extension at 72°C for 10 min. PCR reaction mixtures (20µL total) contained 2µL of 10x PCR buffer, 2µL of dNTP mix (2.5mM each), 1 µL of each primer (10pmoles), 50ng of DNA template, 0.5µL of i-TaqTM DNA polymerase 5U/µL, iNtRON Biotechnology). The resulting PCR product was examined by electrophoresis on a 1.5% agarose gel and purified by PCR quickspin kit (iNtRON Biotechnology). DNA sequencing of the PCR product was done by Macrogen, Seoul. Sequences were blasted to NCBI databases using BlastN and bacterial species identified on the basis of at least 98% similarity to database 16S rDNA sequences.

RESULTS

Table 1: Major Bacterial Species Identified

Gingival crevice	Teeth surface	Tongue surface
Streptococcus	*Streptococcus*	*Streptococcus*
Pneumoniae	*oralis*	*pneumoniae*
Streptococcus	*Streptococcus*	*Streptococcus*
Mitis	*sanguinis*	*mitis*
Streptococcus	*Actinomyces*	*Streptococcus*
Oralis	*viscosus*	*australis*
Actinomyces	*Actinomyces*	*Streptococcus*
Naeslundii	*naeslundii*	*pseudopneumoniae*
Neisseria	*Lautropia sp.*	*Streptococcus*
Subflava		*infantis*
Capnocytophaga granulosa	*Kingella oralis*	*Neisseria subflava*
Pseudomonas Aeruginosa	*Neisseria mucosa*	*Lautropia sp.*
Staphylococcus Aureus	*Neisseria subflava*	
Rothia mucilaginosa	*Rothia mucilaginosa*	

Table 1 shows the highly abundant bacteria from each sampling site. In this study, *S. mitis*, *S. pneumoniae*, *S. oralis*, *S. australis*, *S. infantis*, *S. sanguinis, S. pseudopneumoniae*, *A. naeslundii, A. viscosus, N. subflava, N. mucosa, Capnocytophaga granulosa, Pseudomonas aeruginosa, Staphylococcus aureus, Rothia mucilaginosa, Kingella oralis, Lautropia* sp. have been detected as the major species in the oral cavity using 16S rDNA gene sequencing analysis. Typical 16S sequences obtained are shown in Fig. 1a-c for *C. granulose*, *N. mucosa* and *S. australis* respectively.

GGGGAGCGCAGCTAACATGCAGTGAGGGAGAACCCTTCGGGGCAGAAACC
GGCGCACGGGTGCGTAACGCGTATGCAACCTACCTTTCACAGGGGGATAGC
CCGAAGAAATTTGGATTAATACCCCATAATATTATTAGATGGCATCATTTGA
TAATTAAAATTACCATGGTGAAAGATGGGCATGCGTCCTATTAGCTAGTTG
GAGTGGTAACGGCACCCCAAGGCTACGATAGGTAGGGGTCCTGAGAGGGA
GATCCCCCACACTGGTACTGAGACAGGGACCAGACTCCTACGGGAGGCAGC
AGTGAGGAATATTGGTCAATGGTCGGAAGACTGAACCAGCCATGCCGCGTG
CAGGAAGAATGCCTTATGGGTTGTAAACTGCTTTTATATGGGAAGAATAAG
GAGTACGTGTACTTTGATGACGGTACCATATGAATAAGCATCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGAGGATGCGAGCGTTATTCGGAATCAT
TGGGTTTAAAGGGTCTGTAGGCGGGCTATTAAGTCAGGGGTGAAAGGTTTC
AGCTTAACTGAGAAATTGCCTTGGATACTGGTAGTCTTGAATATCTGTGAA
GTTCTTGGAATGTGTAGTGTAGCGGTGAAATGCTTAGATATTACACAGAAC
ACCGATTGCGGAGGCAGGGGACTAACAGACGATTGACGCTGAGAGACGAA
AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACG
ATGGATACTAGCTGTTTGGAGTAATTTGAGTGGCTAAGCGAAAGTGATAAG
TATCCCACCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGG
AACCTTACCAAGGTTTAAATGGAGACTGACAGGGGCAGAGATGCCTTTTTC
TTCGGACAGTTTTCAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGG
TGTCAGGTTAAGTCCTATAACGAGCGCAACCCCTATTGTTAGTTACCAGCAC
GTGATGGTGGGGACTCTAGCAAGACTGCCGGTGTAAACCGTGAGGAAGGT
GGGGATGACGTCAAATCATCACGGCCCTTACATCTTGGGCTACACACGTGC
TACAATGGTCGTTACAGAGAGCAGCCACTGCGCGAGCAGGAGCGAATCTAT
AAAGACGGTCACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCTG
GAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCAAGCCATGGAAGCTGGGAGTACCTAAGTCGG
TGCCGCAAGGAGCTCCTAGGAATCCGAGC

Fig. 1a: 16S rRNA gene sequence of *Capnocytophaga granulosa* isolated from gingival crevice of subject

GGGGAGCGCGTCTTACTGCAGTCGGACGGCGCACAGAGAAGCTTGCTTCTTGGGTGGCGAG
TGGCGAACGGGTGAGTAACATATCGGAACGTACCGAGTAATGGGGGATAACTAATCGAAAG
ATTAGCTAATACCGCATATTCTTTGAGGAGGAAAGCAGGGGACCTTCGGGCCTTGCGTTATT
CGAGCGGCCGATATCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAG
TAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTC
TGAAGAAGGCCTTCGGGTTGTAAAGGACTTTTGTCAGGGAAGAAAAGGCTGTTGCTAATAT
CGACAGCTGATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGAGCGCAGACG
GTTACTTAAGCAGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCGTTCTGAACTGGGTG
ACTAGAGTGTGTCAGAGGGAGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTG
GAGGAATACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGTTCATGCTCGAAAGCG
TGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAATTAGCTGT
TGGGCAACTTGATTGCTTAGTAGCGTAGCTAACGCGTGAAATTGACCGCCTGGGGAGTACG
GTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGA
TTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATGTACGGAATTCTCCAGAGAC
GGAGGAGTGCCTTCGGGAGCCGTAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTAAGTTG
GGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTC
ATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACATGAGGGTAGCCAAG
CCGCGAGGTGGAGCCAATCTCACAAAGCCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCG
GGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGGGATACCAGAAGTAGGTAGGGAA
CCGCAAGGAGCCCGCTACCACGTGTCTGAGTT

Fig. 1b: 16S rRNA gene sequence of *Neisseria mucosa* isolated from tooth surface of subject

TTTTNNNGGAGCGCGGCTAACTGCAGTAGACGCTGAAGGAAGGAGCTTG
CTCTTTCCGGATGAGTTGCGAACGTTTGAGTAACGCGTAGGTAACCTGCC
TGGTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAG
TAGATGTTGCATGACATTTACTTGAAAGGTGCAAATGCACCACTACCAG
ATGGACCTGCGTTGTATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAA
TGGACGGAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCG
GATCGTAAAGCTCTGTTGTAAGAGAAGAACGAGTGTGAGAGTGGAAAGT
TCACACTGTGACGGTATCTTACCAGAAAGGGACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTCCCGAGCGTTATCCGGATTTATTGGGC
GTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCTGTGGC
TTAACCATAGTACGCTTTGGAAACTGTTTAACTTGAGTGCAGAGGGGGA
GAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAAC
ACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCTCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAGGTGTTGGGTCCTTTCCGGGACTCAGTGCCGCAGCTA
ACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGCTCT
AGAGATAGAGCTTTCCTTCGGGACAGAGGTGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCCTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGC
CGGTAATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGCTGGTACAACGAGTCGC
AAGTCGGTGACGGCAAGCTAATCTCTTAAAGCCAGTCTCAGTTCGGATTG
TAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGAT
CAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC
ACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTACCTTTGGAGC
CGCCGCTAAGTGATGATGGNCATGCCCAA

Fig. 1c: 16S rRNA gene sequence of *Streptococcus australis* isolated from tongue surface of subject

DISCUSSION

Traditionally, oral bacteria have been studied by culture-dependent methods. Identification of the bacteria from culturing method relies on the characteristics observed in biochemical and physical properties of the known and reference strains under optimum growth condition. However, phenotypic characteristics can change under some circumstances like stress, even successful culturing does not necessarily give the correct identification. In comparison, 16S rDNA gene sequencing is more effective and reliable in identification of cultivable bacteria. 16S rRNA gene sequencing will be able to identify atypical phenotypes, rare isolates or poorly studied bacteria regardless of their physiologic characteristics. While most of the baterial strains identified in this present study are commonly found in the oral cavity, some are pathogenic streptococci which have tremendous health implications.

S. mitis and *S. sanguinis* are associated with dental caries, bacterial endocarditis and other serious infections [5,6,14,] while *S. australis* along with *S. infantis* are early colonisers of the oral cavity[15]. *S. oralis* has also been implicated in endocarditis while in immunocompromised patients it may be responsible for septicaemia[3].

Of interest is the presence of pathogenic bacteria such as *S. pneumoniae*, the causative agent for community-acquired pneumococcal pneumonia. While *S. pneumoniae* is a major cause of infections of the respiratory tract, central nervous system and bloodstream, it is also a nosocomial pathogen. Furthermore, antibiotic resistant strains of this pathogen are common[13]. In recent years it has emerged as an important pathogen in HIV-infected patients and immunocompromised subjects[7].

CONCLUSION

The identification of oral bacteria by 16S rDNA gene sequencing is a rapid and reliable method that could profile bacteria of the oral cavity including potential pathogens.

ACKNOWLEDGMENT

The authors wish to thank University of Malaya and the Malaysian Ministry of Science, Technology & Innovation for facilities and the award of research grant E-science 12-02-03-2076 (8422076) to carry out this project.

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