歯の喪失と歯科疾患

特集

ライフスタイルと歯周病の予防

Periodontal disease as lifestyle-related disease and its prevention

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Key words　ライフスタイル　歯周病　予防　歯の喪失

Ⅰ．歯の喪失とその原因

最近，高齢者が健康で心豊かな生活を送るために歯や口の機能の重要性が再認識されてきている．特に，齲蝕および歯周病に代表される歯科疾患は歯の喪失の原因となり，歯が喪失すると食生活や社会生活に支障をきたし，全身の健康にも影響を与え，QOLの低下を招くといわれている．

わが国での歯の喪失状況は，40歳以前はあまり歯の喪失はないが，40歳以降徐々に増加し，さらに，60歳から80歳にかけて急激に歯が失われ，80歳では８歯しか残っていないのが現状である．しかし，平成５年から平成11年の６年間の歯の平均寿命の変化をみると，大臼歯の平均寿命は約５年も延びており，歯の喪失の状況は大幅に改善してきている．また，歯の喪失の原因は齲蝕と歯周病であるが，最近，その比率が大きく変化している．例えば，平成５年では齲蝕が歯の喪失の原因の46％を占め，歯周病の46％と同じ割合であったのが，平成15年では齲蝕は30％に減少したのに対して，歯周病の割合は56％に上昇したという（図1）[1]．したがって，今後，さらに歯の喪失を予防していくためには歯周病を防ぐことが最重要課題である．

Ⅱ．歯周病の現状とそのリスク

歯周病は歯面上に付着したプラーク細菌によって生じる歯肉炎として発症し，さらに，炎症性変化が歯根膜・歯槽骨へ及んで歯周炎へと進行す

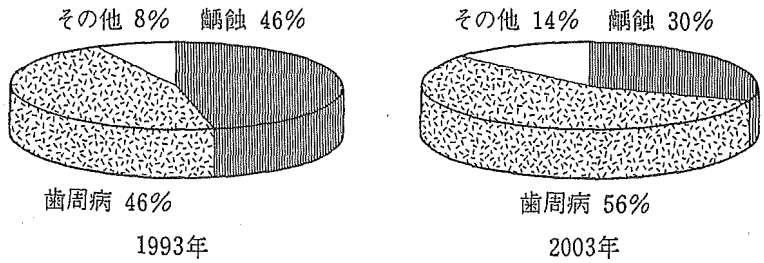

図１　抜歯の原因
（大森ほか，2003[1]）

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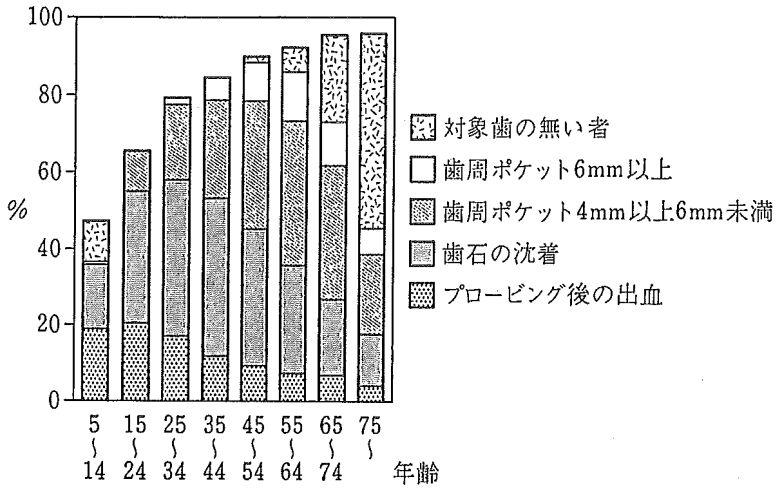

図2 歯周病の有病状態(年齢階級別)
(平成11年 歯科疾患実態調査)

る．一般に，歯周病は慢性に経過するため，強い自覚症状はみられないが，歯肉炎の段階では歯肉の軽度な発赤，腫脹や歯肉出血が認められ，歯周炎に進行すると歯周ポケットからの排膿や歯肉膿瘍の形成がみられ，重度になると歯の動揺をきたし，最終的には歯が抜ける．図2は平成11年歯科疾患実態調査(旧厚生省)での日本人の歯周病の有病状態を示している．プロービング後の出血や歯石の沈着は歯肉炎を示しており，歯周ポケット4mm以上6mm未満は初期から中等度歯周炎，そして，歯周ポケット6mm以上は重度歯周炎といえる．5～14歳の子供でも40%近くが歯肉炎に罹患しており，25～34歳で歯肉炎の有病者が最も多くなる．その後は歯周炎へと進行していき，35～64歳では約80%の人に何らかの歯周病の症状がみられ，45～64歳の約15%の人が重度歯周炎に罹患している．55歳以上では歯周病有病者が減少していくが，これは歯の喪失の結果であって，病状が軽減したためではない．

従来，歯周病の病因論は，宿主と病因との関係，特に歯周病に関連する細菌の面から論じられ，その予防は口腔清掃や歯石除去などにのみ重点が置かれてきた．図3に歯周病に関連するリスクファクターをあげる．歯垢や歯石といった歯周組織を直接破壊する病原要因や，歯周組織の防御・修復にかかわる免疫，遺伝やストレス感受性などの宿主側の種々の要因はもちろんのこと，生活習慣，や社会の医療・保健システムなどの環境要因もリスクファクターとして歯周病の発病・進行の決定的な要因になりうるのである．生活習慣には食生活，喫煙や飲酒といったものが含まれる．したがって，歯周病は長期間にわたる日常生活にかかわるさまざまな要因が影響する生活習慣病であるという認識をもつ必要がある．

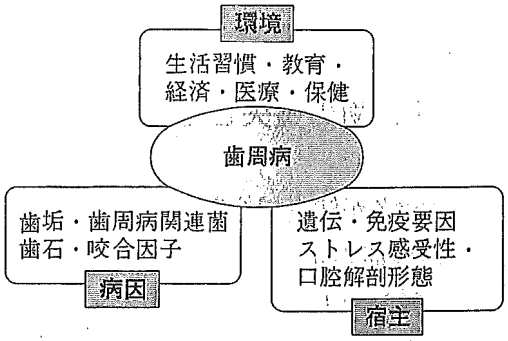

図3 歯周病とリスクファクター

Ⅲ．歯周病に関連するライフスタイル要因

歯周病にはさまざまなライフスタイル要因が関連するといわれている．われわれも，某企業メーカーの従業員を対象に歯周診査とライフスタイルに関する質問票による調査を行い，その結果，肥満，喫煙や飲酒が歯周ポケット4mm以上を有す

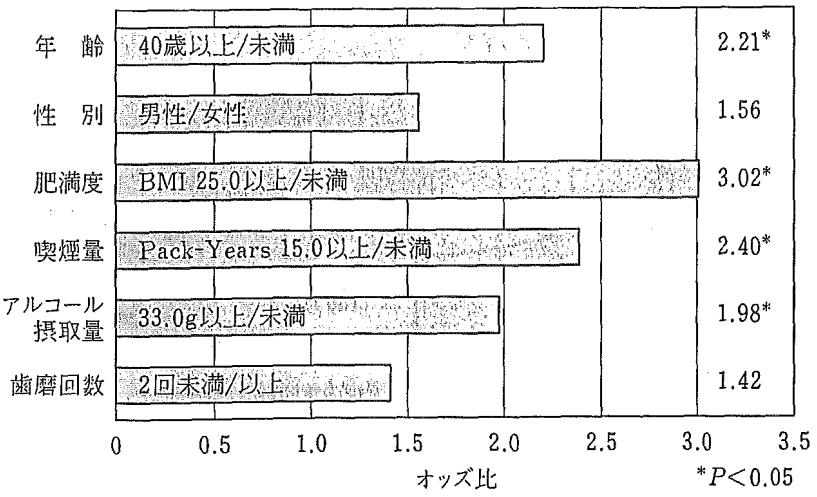

図4　生活習慣が歯周病に及ぼす影響
(Nishida et al. 2004[2])

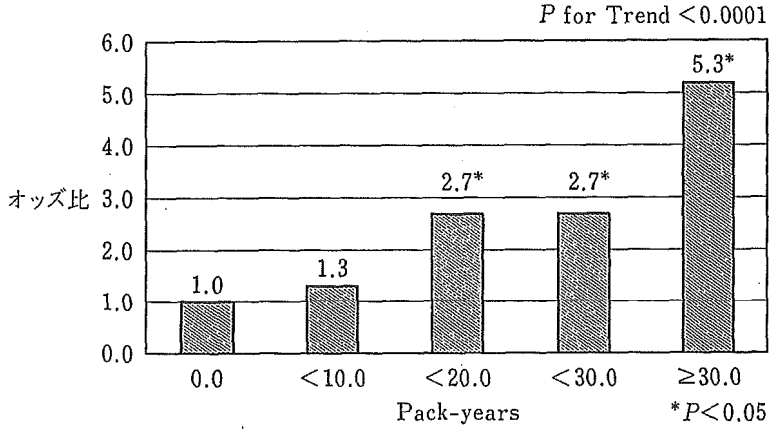

図5　喫煙量と歯周病との関連性
(Nishida et al. submitted[3])

る歯周病の有病歯率と関連していることを明らかにし，歯周病が生活習慣病であることを示した(図4)[2]．さらに，回帰木解析法でリスクの強さをみたところ，ライフスタイル要因の中で喫煙が最も強く，次いで肥満であった[3]．

1. 喫　　煙

喫煙は歯周病の最大のリスクファクターのひとつといわれている．世界各地で行われた多くの調査では，歯周病に対する喫煙のリスクのオッズ比は2～14であると報告されている．また，生涯喫煙量が増えるに従い，歯周病の程度も悪化し，pack-yearsが30以上の人は非喫煙者に比べて5倍以上のリスクがあり，量-反応関係がみられる(図5)[3]．歯周病に罹患している者のなかで，何%の者が喫煙が原因で歯周病になっているのかを示す指標として集団寄与危険度が用いられる．アメリカでの大規模な疫学調査のデータを用いて，歯周病有病者の42%(640万人)が現在吸っているタバコで，11%(170万人)が以前に吸っていたタバコが原因で歯周病に罹患したと推定している(図6)[4]．

一方，禁煙することにより，歯周病のリスクが低下することが示されており，11年以上禁煙を続けると，そのオッズ比は1.2まで低下し，非喫煙者と同じレベルとなり，喫煙の影響がほぼなくなることを示している(図7)[4]．また，喫煙習慣を調べた後，数年後に歯周病の進行を診査したコホート研究などでも，喫煙がリスクとなって，歯周ポケットや歯槽骨の吸収を進行させることが証

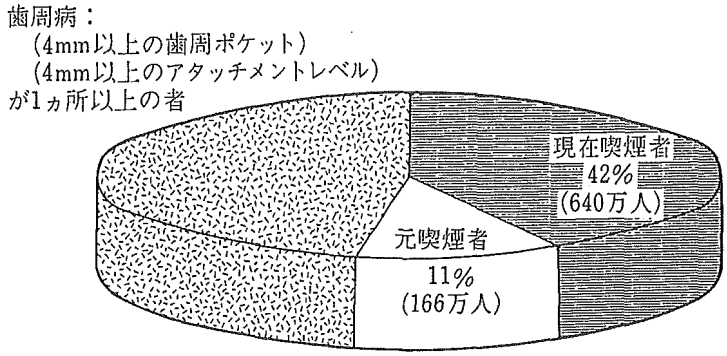

図6 歯周病に対する喫煙習慣の集団寄与危険度
(Tomar & Asma 2000[11])

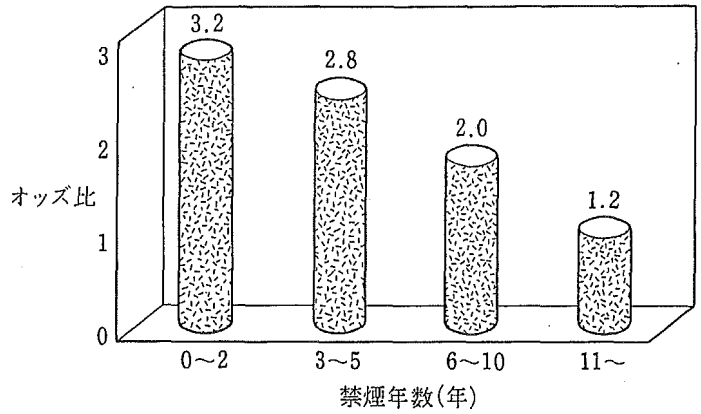

図7 禁煙年数と歯周病との関連性
(Tomar & Asma 2000[11])

明されている．喫煙が歯周組織に及ぼす影響については，①喫煙者の歯肉の微小循環形態や機能が障害されている，②タバコ煙やニコチンは多形核白血球や単球，好中球などの口腔の免疫担当細胞の機能を障害する，③早期発症型歯周炎の喫煙患者では，血清IgGサブクラスが減少している，④ニコチンは歯根表層に沈着し，歯根膜線維芽細胞の機能を障害する，⑤喫煙者ではいくつかの歯周病原性細菌が増加している，⑥喫煙者の歯肉ではフリーラジカルへの反応が高まっている，などがあげられている．喫煙者の歯周病の臨床的特徴は非喫煙者に比べて，ブラッシング時の出血が少なく，歯肉の炎症が軽度であるが，歯槽骨の吸収やアタッチメントロスが大きい．また，喫煙は歯周治療の予後に対しても悪影響を及ぼすことが知られている．喫煙者では歯周治療直後も，メインテナンス期においてもアタッチメントロスの獲得が少なく，この傾向は治療方法に関係なく同じであったという．

2. 飲　　酒

最近，過度の飲酒も歯周病のリスクとなることが報告されている[2)5)]．われわれも，飲酒量と歯周病との関連性を調べたところ，飲酒量と虚血性心疾患と同様にJ字状の関係がみられ，アルコール量33g/日(1.5合)以上を毎日飲む習慣をもつ人が歯周病のリスクが高いことを認めた．しかも図8に示すようにアセトアルデヒドを分解する酵素であるアルデヒド脱水素酵素の遺伝子型の違いで比較すると，強い酵素活性をもつ$ALDH_2$1/1型の人は33g/日以上飲酒していても歯周病の程度に差はみられないが，弱い酵素活性しかもっていない$ALDH_2$1/2型で33g/日以上飲酒している人は，飲酒しない人に比べて，歯周病有病歯率が有意に高いことが示された．アメリカでの大規模なコホート研究でも飲酒習慣と歯周病の発症とが関連して

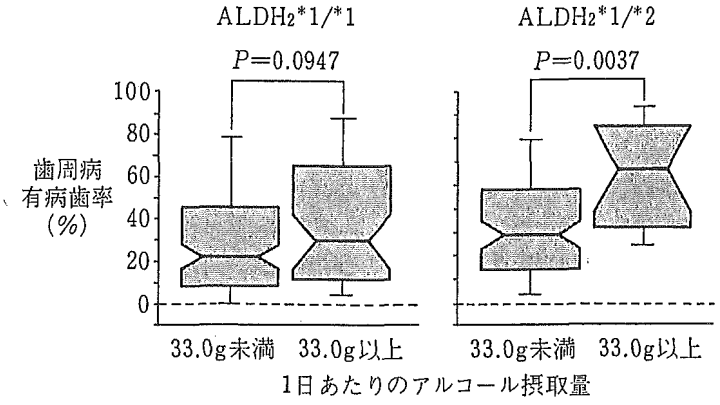

図8 歯周病有病と $ALDH_2$ 遺伝子およびアルコール摂取量との関連性
(Nishida et al. 2004[2])

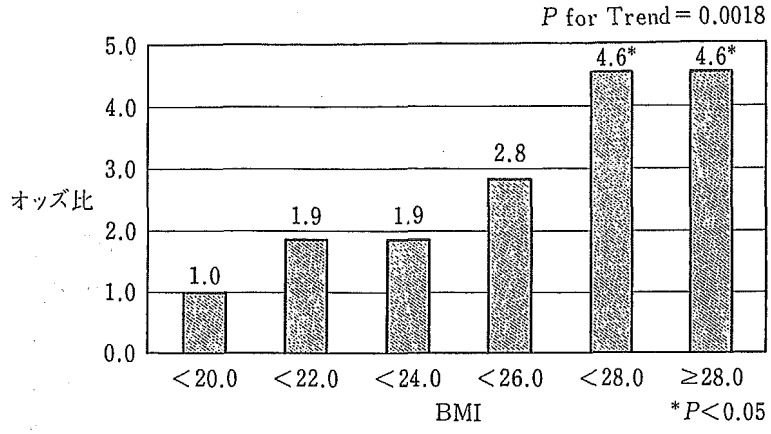

図9 肥満度と歯周病との関連性
(Nishida et al. submitted[3])

いると報告されている[5].

3. 肥　　満

図4にも示したが，肥満が歯周病の発症・進行に影響を及ぼすことが明らかとなってきた[6]. BMI と歯周病の程度の間には量-反応関係がみられ(図9)[3]，また，特に，上半身肥満が歯周病と関連することも報告されている[7]. 一方，肥満と密接に関連する糖尿病患者に歯周病有病者が多いことは古くから知られており，糖尿病の慢性合併症として，腎症，網膜症，神経症，大血管障害，小血管障害とともに，歯周病は第6番目の合併症であるといわれている[8]. 肥満の状態になると，肥満組織から種々の生理活性物質が分泌され，歯周炎の発病にかかわると考えれらている. 脂肪組織から分泌された TNF-α がインスリン抵抗性に直接関与するとともに，歯槽骨吸収に関与している可能性が考えられる. また，肥満により歯周局所の TNF-α や LPS に対する感受性が高くなったり，マクロファージの機能が低下したりすることにより歯周炎が発病しやすくなるかもしれない. 最近，肥満者の内臓脂肪組織から分泌される PAI-1が歯周組織の微細な血管に血液循環障害が生じる可能性も指摘されている.

4. ストレス

慢性歯周炎とストレスに関する疫学的研究において，歯周病に関連すると考えられるさまざまな要因との関連性を考慮しても，ストレスが独立したリスクファクターであることが示されている[9]. たとえば，35～44歳の男性労働者を対象に歯周病と職業性ストレスとの関連性を調べ，仕事に対する精神的要求度および婚姻状態に有意の関連性が認められた. さらに，歯周病は雇用状態や婚姻状

表1 「歯の健康」の目標(健康日本21)

歯周病の予防	歯の喪失防止
◆40，50歳における進行した歯周炎に罹患している者(4 mm 以上の歯周ポケットを有する者)の割合をそれぞれ3割以上減少させる.	◆80歳における20歯以上の自分の歯を有する者の割合を20%以上に，60歳における24歯以上の自分の歯を有する者の割合を50%以上に増加させる.
◆40，50歳における歯間部清掃器具を使用している者の割合をそれぞれ50%以上に増加させる.	◆定期的に歯石除去や歯面清掃を受けている者の割合を30%以上に増加させる.
◆喫煙が及ぼす健康影響についての知識の普及	◆定期的に歯科健診を受けている者の割合を30%以上に増加させる.
◆禁煙，節煙を希望する者に対する禁煙支援プログラムをすべての市町村で受けられるようにする.	

態に加えて，マイナスのライフイベント(生活出来事)のインパクトが関連するという報告がなされている．また，別の調査によると，慢性歯周炎では，心理的要因との関連性はみられなかったが，成人発症型急速進行性歯周炎患者では抑うつ度と孤独感が強いという結果が得られている．さらに，種々の要因を調整しても経済的ストレスがアタッチメントロスと歯槽骨吸収の，また，抑うつ度がアタッチメントロスのリスクインディケーターとなることが示されている．ストレス対処行動を分析すると，強い経済的ストレスをもっている者のなかで，情動中心対処行動をとる者および問題解決対処行動をとらない者に，アタッチメントロスや歯槽骨吸収に対する高いオッズ比を認めた．しかし，強い経済的ストレスをもつ者でも，適切な対処行動をとる者は歯周病有病との関連性はみられなかったという．

Ⅳ．ライフスタイルからみた歯周病の予防

ライフスタイルからみた歯周病の予防として，①禁煙をする，②肥満を防止する，③過度の飲酒をしない，④ストレスを発散させるなどの予防が考えられる．特に，タバコについては，最近，受動喫煙も歯周病のリスクとなることが報告されており，非喫煙者に対する受動喫煙の防止も重要である．表1に，健康日本21における歯周病の予防と歯の喪失防止の目標を示す[10]．歯周病の予防を考えるとき，セルフケア，プロフェッショナルケアとパブリックヘルスケアからの予防を考えなければならない．セルフケアとしては健康なライフスタイルをもつことは当然であるが，歯間清掃器具の使用など良好な口腔清掃習慣をもつことも欠かせない．また，歯周病に壮年期の約80%の人が罹患していることを考えると，プロフェッショナルケアとして，定期的に健診を受け，歯石除去や歯面清掃を受ける必要がある．今後，さらに，歯周病の一次予防をパブリックヘルスの面から進めるためにも，喫煙をはじめとする健康に関する知識の普及やそれを実践するための環境面からの整備が必要であろう．

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Quantitative detection of periodontal pathogens using real-time polmerase chain reaction with TaqMan probes

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Kuboniwa M, Amano A, Kimura RK, Sekine S, Kato S, Yamamoto Y, Okahashi N, Iida T, Shizukuishi S. Quantitative detection of periodontal pathogens using real-time PCR with TaqMan probes.
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Quantitative analysis, with identification of periodontopathic bacteria, is important for the diagnosis, therapeutic evaluation and risk assessment of periodontal disease. We developed a highly sensitive and specific method using real-time polymerase chain reaction (PCR) to detect and quantify six periodontal bacteria: *Porphyromonas gingivalis, Tanerella forsythia, Actinobacillus actinomycetemcomitans, Treponema denticola, Prevotella intermedia*, and *P. nigrescens*. Species-specific TaqMan probe/primer sets were designed according to 16S ribosomal RNA gene sequences. Plaque and tongue debris specimens were collected from 10 patients with advanced periodontitis and 10 periodontal healthy individuals and analyzed. All species, except for *P. nigrescens*, were detected in samples from diseased sites in significantly greater numbers than in those from healthy sites, whereas greater numbers of *P. nigrescens* were found in the controls. These results suggest that the present real-time PCR method with the designed probe/primer sets enabled sensitive detection of the six periodontal bacteria, and may also assist future microbial studies of periodontal diseases.

Key words: *Actinobacillus actinomycetemcomitans*; *Porphyromonas gingivalis*; *Prevotella intermedia*; *Prevotella nigrescens*; quantitative detection; real-time polymerase chain reaction; *Tanerella forsythia*; TaqMan probe; *Treponema denticola*

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Periodontal diseases are infectious disorders and the pathogenic microbial populations involved are known to be highly complex. Numerous reports have demonstrated a close association between periodontitis and a small subset of microbial species that includes *Porphyromonas gingivalis, Tanerella forsythia, Actinobacillus actinomycetemcomitans, Treponema denticola*, and *Prevotella intermedia* (12, 22, 47, 49, 53). These pathogens are harbored on the tongue surface and their metabolic products have also been suggested as causative factors of halitosis (11, 38, 43). Therefore, quantitative analysis with identification of pathogens in clinical specimens would be helpful for the diagnosis and therapeutic evaluation of periodontitis, as well as to understand the pathogenesis of halitosis.

Currently, several methods of quantitative analysis are used to identify oral pathogens, including flow cytometry (54), a DNA–DNA hybridization (8, 48, 51), and real-time polymerase chain reaction (PCR) (4, 30, 33, 40, 45, 60, 61). Real-time PCR has some advantages, as its detection limit of approximately 10^2 copies is more sensitive than that of a DNA probe (10^3–10^4 copies) (8, 48). In addition, a real-time PCR assay, along with a universal probe/primer set, can be used to quantify an entire bacterial load in a single clinical specimen with a fair degree of precision, which is not possible with a DNA probe. As for flow cytometry, most bacteria are optically too similar to be distinguished from each other or from debris without artificially modifying the target bacteria with fluorescent labeling techniques, such as fluorescent antibodies or dyes (23, 54). Furthermore, coaggregation of bacteria and the presence of different contaminating matrices (e.g. dirt, food, dental plaque) can also make accurate counting difficult with direct or fluorescence microscopy. For the reasons stated above, real-time PCR would currently be more suitable for quantitative detection of microorganisms than the other methods.

A variety of *Prevotella* species are commonly detected in the human oral cavity

(19, 31, 34), with *P. intermedia* and *P. nigrescens* the most prevalent. *P. intermedia* is considered to be a periodontal pathogen, whereas *P. nigrescens* is a marker of relative periodontal health (7–9, 16, 17, 19, 31, 36, 37, 44). These species are phenotypically very similar, and biochemical or serological differentiation is considerably difficult and laborious (7, 10, 14, 17, 29, 34, 46). In light of the postulated different roles of these two species, however, it is essential to identify and quantify them differentially in clinical specimens. A real-time PCR method using SYBR Green I, a double strand DNA binding dye, for detecting five periodontal pathogens (*P. gingivalis, A. actinomycetemcomitans, T. forsythia, T. denticola* and *T. socranskii*), has been reported (45). In the TaqMan system, a set of three specific PCR probes, forward and reverse primers, and TaqMan probe, is used. The real-time PCR with TaqMan probe allows continuous measurement of products throughout the reaction in a closed tube and exploits the 5' to 3' exonuclease activity of Taq polymerase in conjunction with fluorogenic DNA probes. In this method, a TaqMan probe, designed to hybridize to the target PCR product, is labeled with a fluorescent reporter dye and a quencher dye. During PCR [Q1] amplification, the probe is digested by Taq polymerase, separating the dyes and resulting in an accumulation of reporter fluorescence along with a corresponding increased fluorescence intensity (4, 30, 33, 40, 60, 61). Thus, TaqMan hybridization probes are likely to be adopted as more reliable options for distinguishing between periodontal pathogens, especially for such closely related species as *P. intermedia* and *P. nigrescens*. For the same reason, a TaqMan system would also be useful for distinguishing *A. actinomycetemcomitans* (formerly *Haemophilus actinomycetemcomitans*) from *H. influenzae*, based on their 16s rRNA sequences. In the present study, species-specific TaqMan probe/primer sets were designed for rapid and reliable quantitative identification of *P. gingivalis, T. forsythia, A. actinomycetemcomitans, T. denticola, P. intermedia*, and *P. nigrescens*. Using these probe/primer sets, clinical specimens from individuals with varying clinical conditions were analyzed.

Material and methods

Bacterial strains

P. gingivalis, ATCC33277, ATCC53977, 6/26, HW24D1, W50, and HNA99; *A. actinomycetemcomitans*, ATCC29522, ATCC29523, and FDCY4; *T. forsythia*, ATCC43037; *T. denticola*, ATCC33520; *P. intermedia*, ATCC25611; and *P. nigrescens*, ATCC25261 were used as reference strains. *P. gingivalis*, *P. intermedia*, and *P. nigrescens* cells were grown in trypticase soy broth as described previously (26), while *A. actinomycetemcomitans* was grown in TSB supplemented with yeast extract (1 mg/ml) and sodium bicarbonate (1 mg/ml) (42). *T. forsythia* and *T. denticola* were also grown under conditions described previously (41,59). Each species was cultured at 37°C under anerobic conditions (80% N_2, 10% CO_2, 10% H_2) to the late exponential phase, then harvested by centrifugation (12,000 *g* at 4°C for 2 min) and washed with phosphate-buffered saline (PBS) (pH 7.4).

Species-specific probe/primer sets for real-time PCR

Multiple alignment analyses of the rRNA genes of 84 major oral bacteria were employed as reference materials for designing the assays.

The 16S rRNA gene sequences (GenBank) from the following bacteria were aligned using the Clustal W program accessed from DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/): *Actinomyces bovis, A. israelii, A. naeslundii, A. odontolyticus, A. viscosus, A. delphinicola, A. actinomycetemcomitans, A. seminis, A. suis, Bacteroides forsythus, B. fragilis, Bifidobacterium dentium, Corynebacterium matruchotii, Capnocytophaga gingivalis, C. ochracea, C. sputigena, Campylobacter rectus, C. sputorum, Desulfovibrio* sp., *Eikenella corrodens, Enterococcus faecalis, E. faecium, Escherichia coli, Eubacterium nodatum, E. timidum, Filifacter alocis, Fusobacterium nucleatum* subsp. nucleatum, *F. sulci, F. simiae, Haemophilus aphrophilus, H. ducreyi, H. haemolyticus, H. influenzae, H. paracuniculus, H. parainfluenzae, H. paraphrophilus, H. parasuis, Klebsiella pneumoniae, Leptotrichia buccalis, Lactobacillus casei, Legionella pnuemophila, Neisseria cinerea, N. denitrificans, N. flavescens, N. mucosa, Pasteurella avium, P. multocida, P. pneumotropica, Peptococcus niger, Peptostreptococcus anaerobius, P. micros, P. pervotii, Porphyromonas cansulci, P. endodontalis, P. gingivalis, P. heparinolytica, P. intermedia, P. loescheii, P. melaninogenica, P. nigrescens, P. oralis, P. oris, P. veroralis, Propionibacterium acnes, P. propionicus, Pseudomonas aeruginosa, P. fluorescens, Ruminobacter amylophilus, Salpingitia* sp., *Selenomonas sputigena, Staphylococcus aureus, S. epidermidis, Streptococcus* sp., *S. anginosus, S. bovis, S. constellatus, S. cricetus, S. downei, S. gordonii, S. intermedius, S. lactis, S. macacae, S. mitis, S. mutans, S. oralis, S. pyogenes, S. ratti, S. salivarius, S. sanguis, S. saprophyticus, S. sobrinus, Serratia marcescens, Treponema denticola, T. medium, T. pallidum, T. pectinovorum, T. socranskii* subsp. socranskii, *T. vincentti, Veillonella atypical, V. dispar, V. parvula*, and *Vibrio cholerae*.

Species-specific probe and primer sets were designed from the variable regions of the 16S rRNA gene sequences. Regions of identity were surveyed for possible cross-hybridization with other bacterial genes using the rRNA BLAST program, which was accessed from The European Ribosomal RNA database (http://oberon.fvms.ugent.be:8080/rRNA/), and the BLAST program accessed from National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov./blast/). All probe/primer sets were subjected to Primer Express version 1.0, using the guidelines established by Applied Biosystems (Foster City, CA). A universal probe/primer set was used as described previously (40). The probes and primers were synthesized by Applied Biosystems, except the forward primer of *P. nigrescens* (the mixed primer), which was synthesized by Sigma Genosys (The Woodlands, TX). The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) at the 3' end and stored at −20°C.

Clinical specimens

Seventy plaque samples, whose bacterial profiles were analyzed in our previous study (2), were used to confirm the specificities of the newly designed TaqMan probe/primer sets. In addition, subgingival plaque and tongue surface debris specimens were collected from 20 individuals, of whom 10 were patients with advanced periodontitis (mean age 48.3 ± 15.9 years) and 10 were periodontal healthy controls (mean age 56.6 ± 12.4 years). The periodontitis patients possessed active sites in greater than 40% of all their teeth, which were defined by probing depth of 6 mm or greater, bleeding on probing, and the presence of either erythema or suppuration. The controls had probing depths of 3 mm [Q2] or less and exhibited no clinical signs of [Q3] alveolar bone loss in dentition, bleeding on [Q4,5,6] probing, or signs of erythema or suppuration. The subgingival plaque samples were collected from the deepest pocket of each subject in a manner described previously (2), while tongue surface debris samples

were collected with sterile spatulas as thoroughly as possible and then immediately suspended in sterile PBS. After collection, all samples were kept on ice, and genomic DNA was immediately extracted, as described below.

DNA isolation

Genomic DNA isolation from the specimens was performed using a PUREGENE DNA Isolation Kit (Gentra systems, Minneapolis, MN) according to the manufacturer's instructions. Purified genomic DNA of *H. influenzae* ATCC 33991 was purchased from American Type Culture Collection (ATCC, Manassas, VA). DNA concentrations were determined spectrophotometrically using a GeneQuant II RNA/DNA Calculator (Amersham Pharmacia Biotech, Piscataway, NJ).

Screening by conventional PCR

Bacterial species-specific primers used for conventional PCR are shown in Table 1. PCR amplification was performed in a reaction mixture (25 μl) consisting of Ready-To-Go PCR beads (Amersham Pharmacia Biotech) containing an enzyme and the required reagents, along with 0.8 μM of each primer and 2 μl of the template DNA solution (20–50 μg/ml), as described previously (3). The amplification reaction was performed in a model 2400 thermal cycler (Perkin Elmer, Branchburg, NJ) with the cycling parameters set as follows.

- For *T. forsythia, P. intermedia*, and *P. nigrescens*: an initial denaturation at 95°C for 5 min; 30 cycles consisting of 95°C for 30 s, 55 or 60°C for 30 s and 72°C for 1 min; and a final extension at 72°C for 7 min.
- For *P. gingivalis, A. actinomycetemcomitans*, and *T. denticola*: an initial denaturation at 95°C for 5 min; 30 cycles consisting of 95°C for 30 s, 55 or 60°C for 30 s and 72°C for 45 s; and a final extension at 72°C for 7 min.

The annealing temperature (Ta) varied depending on the primer sets (Table 1). For negative and positive controls, the PCR assays were also performed with or without the isolated genomic DNA from the reference strains of the targeted organisms. The PCR products were subjected to electrophoresis on a 2% agarose gel with Tris acetate EDTA buffer. The gel was stained with 0.5 μg/ml of ethidium bromide and photographed under ultraviolet illumination. An EZ load 100 bp (Bio-Rad, Hercules, CA) was used as the molecular size standard.

Quantitative analysis by real-time PCR

Real-time PCR was carried out using a LIGHTCYCLER™ system (Roche Diagnostics, Mannheim, Germany) and the designated capillaries. Duplicate samples were routinely used for determination. Each PCR was performed in a total volume of 20 μl containing 2 μl of ×10 LIGHTCYCLER-DNA Master Hybridization Probes (Roche Diagnostics), 0.2 μl each of forward and reverse primers (final concentration, 500 nM each), an appropriate dose of the TaqMan probe (final concentration 200 nM; Applied Biosystems), an appropriate amount of $MgCl_2$ (final concentration 3–6 mM), 2 μl of template DNA solution and an appropriate dose of sterilized Dnase–Rnase-free water. The optimized $MgCl_2$ final concentration in each species-specific reaction solution was determined as follows: 4 mM for *P. gingivalis*, 5 mM for *A. actinomycetemcomitans*, 3 mM for *T. forsythia*, 6 mM for *T. denticola*, 3 mM for *P. intermedia*, 4 mM for *P. nigrescens*, and 5 mM for the universal probe/primer reaction solution. Each amplification reaction was performed in the LightCycler with the cycling parameters set as follows.

- For *P. gingivalis, T. forsythia, T. denticola*, and *P. nigrescens*: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 57°C for 15 s, and 72°C for 5 s, and a final cooling at 40°C for 8 min.
- For *P. intermedia*: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 56°C for 15 s and 72°C for 8 s, and a final cooling at 40°C for 8 min.
- For *A. actinomycetemcomitans*: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 57°C for 15 s and 72°C for 35 s, and a final cooling at 40°C for 8 min.
- For Universal: an initial denaturation at 95°C for 1 min, 50 PCR cycles at 95°C for 5 s, 58°C for 15 s and 72°C for 20 s, and a final cooling at 40°C for 8 min.

Fluorescence intensity was monitored at the annealing temperature in single acquisition mode. The dye signals generated during a run were measured in fluorimeter channel 2 (F2, 640 ± 30 nm) and channel 1 (F1, 530 ± 30 nm) and the results were indicated as the F2/F1 ratio, which was considered adequate for a TaqMan probe conjugated with FAM and TAMRA. Fluorescent data were analyzed with LightCycler Data Analysis (LCDA) software version 3.5 (Roche Diagnostics).

Table 1. Species-specific primers for conventional PCR

Primer sets	Product size (bp)	Ta (°C)*	Reference
Porphyromonas gingivalis 5′-TGTAGATGACTGATGGTGAAAACC-3′ 5′-ACGTCATCCCCACCTTCCTC-3′	197	60	(35)
Actinobacillus actinomycetemcomitans 5′-CTAGGTATTGCGAAACAATTTG-3′ 5′-CCTGAAATTAAGCTGGTAATC-3′	262	55	(20)
Tanerella forsythensis 5′-GCGTATGTAACCTGCCCGCA-3′ 5′-TGCTTCAGTGTCAGTTATACCT-3′	641	60	(5)
Treponema denticola 5′-TAATACCGAATGTGCTCATTTACAT-3′ 5′-TCAAAGAAGCATTCCCTCTTCTTCTTA-3′	316	55	(45)
Prevotella intermedia 5′-TTTGTTGGGGAGTAAAGCGGG-3′ 5′-TCAACATCTCTGTATCCTGCGT-3′	575	55	(5)
Prevotella nigrescens 5′-ATGAAACAAAGGTTTTCCGGTAAG-3′ 5′-CCCACGTCTCTGTGGGCTGCGA-3′	804	55	(5)

*Annealing temperature (Ta) of PCR reaction.

Calculation of theoretical cell numbers by real-time PCR

The bacterial DNA levels were quantified by real-time PCR and converted to theoretical cell numbers by the following method. TaqMan technology provided by the manufacturer determines the PCR cycle at which the increase in fluorescence of the reporter dye reaches a threshold cycle (C_T). C_T is proportional to the log of the amount of the target gene and, hence, the log of the number of bacteria in the sample, provided there are the same copy numbers of the reported sequence within a genome of various bacteria. When using a gene as a detecting target for the

accurate quantification of cell numbers of certain bacteria (not only in real-time PCR, but also in DNA hybridization), the genome weight and targeted gene copy numbers per cell must be known, though they are also affected by doubling time. There are numerous types of bacteria in the oral cavity and it is impossible to accurately know each of their genome weights and 16s rRNA copy numbers. In the present study, therefore, the total bacterial load in the clinical specimens was calculated on the assumption that the 16s rRNA gene copy numbers of the oral anaerobes were not significantly different from each other (22, 40). *P. gingivalis*, whose genome has recently been sequenced and for which the exact genome size (2.2 Mb) and weight (2.37 fg) are known (33), was used as the representative bacterium for the reasons mentioned above.

First, serial dilutions of *P. gingivalis* genomic DNA were analyzed using the universal probe/primer set. Serial dilutions of the other five bacterial genomic DNA were then analyzed using the universal probe/primer set and their theoretical cell numbers calculated based upon standard curves derived from the genomic DNA of *P. gingivalis*. Adjusted in this manner, serial dilutions comparable to 10^2–10^7 colony-forming units were used for quantitative specific detection of each targeted pathogen.

Statistical analysis

A Mann–Whitney *U*-test was used for comparative analysis among groups of clinical specimens.

Results

Design of the species-specific probe/primer sets

A multiple alignment among the 16S rRNA gene sequences of 84 oral bacteria was performed using Clustal W. The species-specific probe/primer sets were designed according to the sequence of identity determined with Primer Express ver.1 (Table 2). The specificities of the newly designed probes and primers were further confirmed by multiple alignment of the relevant sequences of closely related species and a BLAST homology search program (NCBI). Probe/primer sets for *P. intermedia* and *A. actinomycetemcomitans* were designed according to the sequences of the complementary strand. The 16S rRNA sequences of the closely related *P. intermedia* and *P. nigrescens* showed a high similarity (92% homology), thus, distinctly variable regions between these two sequences were successfully identified for designing the species-specific probe/primer sets (Fig. 1).

Quantitative sensitivity of species-specific probe/primer sets

The total number of bacterial cells was determined using the TaqMan PCR procedure with the universal probe/primer set. The standard curve was analyzed with the universal probe/primer set against a serial dilution of *P. gingivalis* genomic DNA, which corresponded to 10^2–10^7 cells and showed a credible error value (error = 0.0707) (Fig. 2). We confirmed the weight of the purified genomic DNA of *P. gingivalis*, which corresponded to 10^7 cells by the colony counting method and compared it to the genomic weight calculated by the hypothesis employed in this study. The former amount was approximately 15% less than the latter. Serially diluted genomic DNA solutions purified from each of the six pure bacterial species cultures, which were converted to number of cells, were employed for analyzing the standard curve used for quantitative detection with the newly designed probe/primer sets. The

Table 2. Species-specific primers /TaqMan probes for real-time PCR

Primers/TaqMan Probe sets	Length	Tm*	%GC	Product size (bp)	Ta (°C)	Reference
Universal						
5'-TCCTACGGGAGGCAGCAGT-3'				466	58	(33)
5'-GGACTACCAGGGTATCTAATCCTGTT-3'						
5'-FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA-3'						
Porphyromonus gingivalis						
5'-ACCTTACCCGGGATTGAAATG-3'	21	59	48	83	57	This study
5'-CAACCATGCAGCACCTACATAGAA-3'	24	60	46			
5'-FAM-ATGACTGATGGTGAAAACCGTCTTCCCTTC-TAMRA-3'	30	69	47			
Actinobacillus actinomycetemcomitans						
5'-CCCATCGCTGGTTGGTTA-3'	18	56	56	696	57	This study
5'-GGCACGTAGGCGGACC-3'	16	57	75			
5'-FAM-CCTCTGTATACGCCATTGTAGCACGTGTGT-TAMRA-3'	30	68	50			
Tanerella forsythensis						
5'-AGCGATGGTAGCAATACCTGTC-3'	22	57	50	88	57	This study
5'-TTCGCCGGGTTATCCCTC-3'	18	59	61			
5'-FAM-TGAGTAACGCGTATGTAACCTGCCCGC-TAMRA-3'	27	70	56			
Treponema denticola						
5'-CCGAATGTGCTCATTTACATAAAGGT-3'	26	60	38	122	57	This study
5'-GATACCCATCGTTGCCTTGGT-3'	21	60	52			
5'-FAM-ATGGGCCCGCGTCCCATTAGC-TAMRA-3'	21	70	67			
Prevotella intermedia						
5'-TCCACCGATGAATCTTTGGTC-3'	21	58	48	98	56	This study
5'-ATCCAACCTTCCCTCCACTC-3'	20	57	55			
5'-FAM-CGTCAGATGCCATATGTGGACAACATCG-TAMRA-3'	28	69	50			
Prevotella nigrescens						
5'-CCGTTGAAAGACGGCCTAA-3'	19	57	53	82	57	This study
5'-CCCATCCCTTACCGGRA-3'	17	55	59			
5'-FAM-CCCGATGTGTTTCATTGACGGCATC-TAMRA-3'	25	69	52			

*Melting temperature of DNA (Tm) was analyzed with Primer Express version 1.0 (Applied Biosystems).

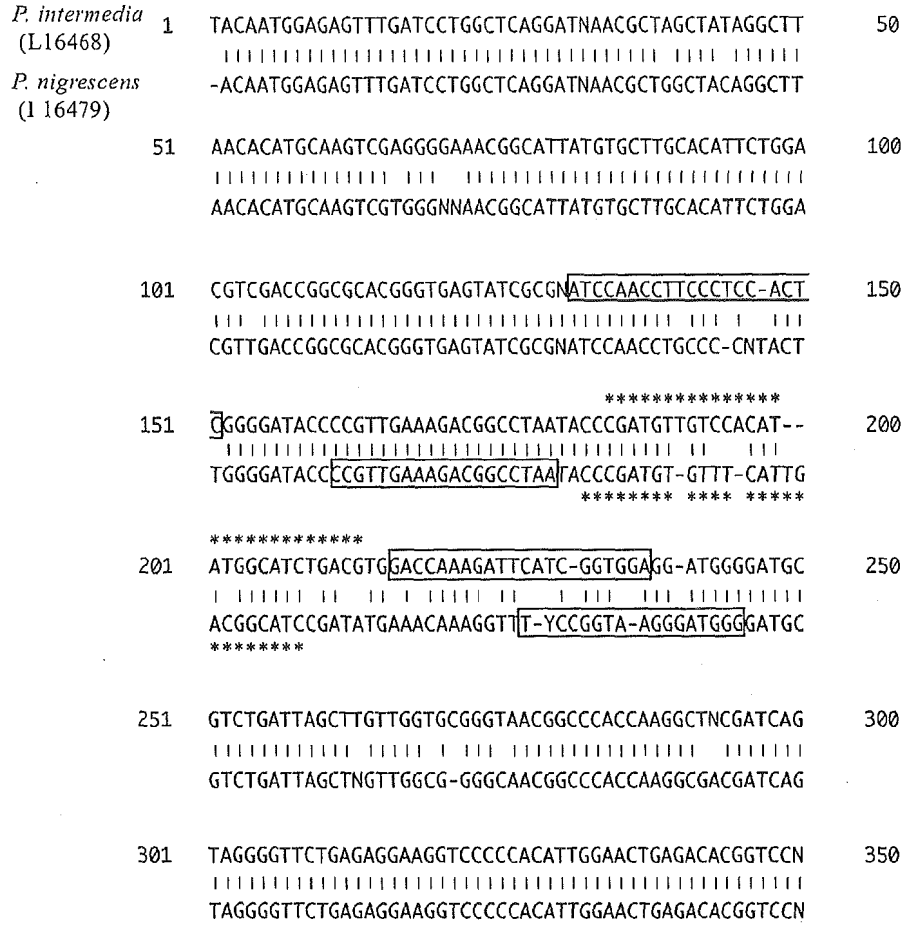

Fig. 1. Maximum matching analysis between the partial 16S ribosomal RNA gene sequences of *P. intermedia* and *P. nigrescens*. Maximum matching analysis between partial 16S rRNA genes of these two closely related species was performed using DNASIS-MAC version 3.7 (Hitachi Software Engineering, Tokyo, Japan), and variable regions were identified for designing the species-specific probe/primer sets. Dotted sequences indicate TaqMan hybridization probes, and boxed sequences show the forward and reverse primers. The probe/primer set for *P. intermedia* was designed according to the sequence of the complementary strand.

curve of each pathogen between logarithms of serially diluted genomic DNA and threshold PCR cycles was found to be linear over a wide range, corresponding to 10^2–10^7 cells (*P. gingivalis*, error = 0.0695; *A. actinomycetemcomitans*, error = 0.0288; *T. forsythia*, error = 0.0354; *T. denticola*, error = 0.0311; *P. intermedia*, error = 0.0898; and *P. nigrescens*, error = 0.0182) (Fig. 2).

Specificity of species-specific probe/primer sets

The newly designed species-specific probe/primer sets showed no cross-reactivity with any other species when used with the DNA samples of the reference strains and clinical samples of bacterial profiles from our previous study (data not shown) (2). In addition, there was no cross-reactivity of the probe/primer set for *A. actinomycetemcomitans* with *H. influenzae* (data not shown). Further, the differential identification and quantification of *P. intermedia* and *P. nigrescens* were confirmed. The determined amounts of *P. intermedia* DNA were accurately quantified by real-time PCR, even in the presence of the same amount of *P. nigrescens* genomic DNA (Fig. 3a). The quantitative detection of *P. nigrescens* genomic DNA was not affected by *P. intermedia* DNA (Fig. 3b).

Crude genomic DNA samples were extracted from 10 subgingival plaque (P-pocket) and 10 tongue surface debris (P-tongue) specimens taken from patients with advanced periodontitis, as well as from 10 subgingival plaque (H-pocket) and 10 tongue surface debris (H-tongue) specimens from periodontal healthy individuals, and analyzed (Table 3). The identification and quantification of theoretical cell numbers of periodontal bacteria were estimated by real-time PCR, and their specific reactivity with the targeted species was again demonstrated by a conventional PCR assay.

Comparison of proportion of periodontal bacteria in clinical specimens

The prevalence and amount of the targeted species harbored by the subjects were compared using a real-time PCR assay (Fig. 4). In the subgingival samples, all periodontal pathogens, except for *P. nigrescens*, were more prevalent in the diseased sites than in the healthy sites and in a significantly greater proportion in diseased pockets ($P < 0.05$). *P. nigrescens* was detected in greater proportions in the controls. In the tongue debris samples, greater proportions of *P. gingivalis* ($P = 0.0051$), *P. intermedia* ($P = 0.0342$), and *T. denticola* ($P = 0.0051$) were found in the patients than in the non-disease controls, but no significant differences were found in the

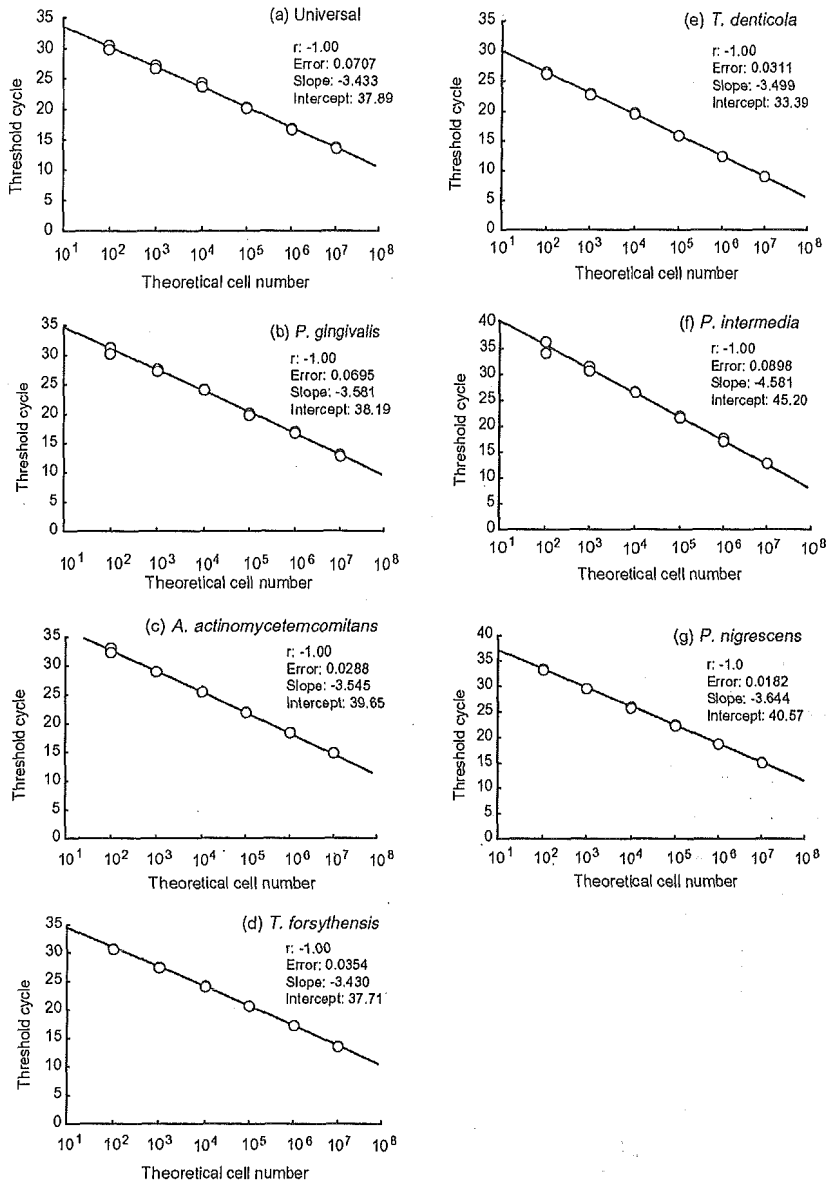

Fig. 2. Correlation between threshold cycle and number of cells. Standard curves from real-time PCR with the universal or species-specific probe/primer sets are shown. The threshold cycle is the cycle number when threshold fluorescence was reached. The theoretical cell number was calculated as described in Material and Methods. Reactions of the universal or species-specific probe/primer sets with genomic DNA of the targeted pathogen are shown. a) Universal with *P. gingivalis* DNA. Species specific with (b) *P. gingivalis*, (c) *A. actinomycetemcomitans*, (d) *T. forsythia*, (e) *T. denticola*, (f) *P. intermedia*, and (g) *P. nigrescens*. Each reaction was performed in duplicate. By plotting the standard curve values, using LIGHTCYCLER software version 3.5.28, we generated the represented data. 'Slope' represents the overall reaction efficiency. PCR efficiency (E) was calculated by the following formula; $E = 10^{-1/slope}$. 'Error' (mean squared error) provided clues to tube to tube variations, e.g. pipetting errors. The standard curve was considered reliable when the error value was <0.2. 'Intercept' is the value of y-intercept and '*r*' is the correlation coefficient.

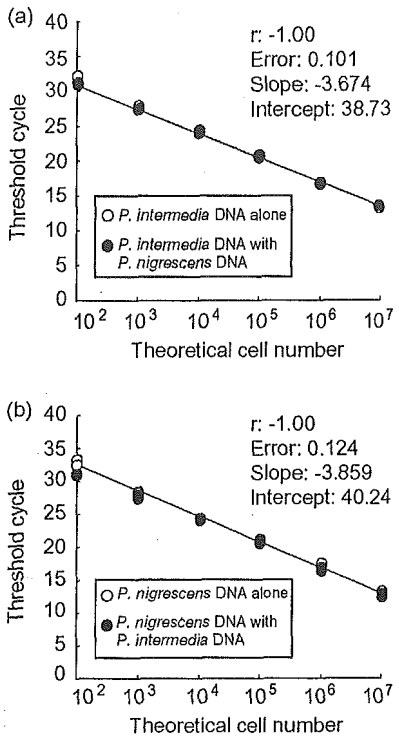

Fig. 3. Specific detection of *P. intermedia* and *P. nigrescens* using real-time PCR. We confirmed the differential identification and quantification of *P. intermedia* and *P. nigrescens*. Serial dilutions of genomic DNA from *P. intermedia* and *P. nigrescens*, corresponding to 10^7–10^2 cells (16.6 ng to 166 fg in *P. intermedia*, and 8.74 ng to 874 fg in *P. nigrescens*), were used as templates for real-time PCR. The reactions were performed with the targeted bacterial DNA in the presence or absence of another equivalent DNA template. Each assay was repeated twice. a) Standard curve from real-time PCR with *P. intermedia* specific probe/primer set. b) Standard curve from real-time PCR with *P. nigrescens* specific probe/primer set.

proportions of *A. actinomycetemcomitans*, *T. forsythia*, and *P. nigrescens*. When we analyzed the presence or absence of the target species in samples from patients, the existing bacteria in the plaque samples were shown to be in the tongue debris taken from the same subject (Table 3; Fig. 4).

Discussion

The bacterial 16S rRNA gene is a useful target for detection and quantification of bacteria in a variety of complex environmental and health-related situations, during which a multi-species population is sampled along with impurities, or where the bacteria are internalized within a matrix (1, 21, 23, 55–57). In theory, the variable regions of the 16S rRNA gene should provide a means for species-specific detection and enumeration of complex bacterial populations by real-time PCR (24, 32, 33, 40, 55–57). Therefore, once species-specific TaqMan probe/primer sets are successfully designed for pathogens, a real-time PCR assay can be used as a reliable tool for rapid and highly sensitive enumeration. In the present study, multiple alignments of the 16S rRNA gene sequences of 84 oral bacterial species of interest were analyzed. The quantitative sensitivity and specificity of newly designed TaqMan probe/primer sets were verified using both reference strains and clinical specimens, for which the presence

Table 3. Analysis of bacterial distribution in subgingival plaque and tongue debris specimens by conventional and real-time PCR

	Universal	*P. gingivalis*		*A. actinomycetemcomitans*		*T. forsythensis*		*P. intermedia*		*P. nigrescens*		*T. denticola*	
Sample no.*	Real-time PCR**	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR
P-pocket-1	2.86E+07	+	6.59E+02	+	8.18E+03	+	5.65E+04	+	1.22E+06	+	5.81E+04	+	8.53E+04
P-pocket-2	4.77E+07	+	8.04E+06	−	−	+	1.07E+06	+	1.94E+07	+	<100	+	3.87E+05
P-pocket-3	6.28E+07	+	1.15E+06	+	9.33E+04	+	4.15E+05	+	7.24E+06	+	3.31E+02	+	1.74E+05
P-pocket-4	1.99E+07	+	<100	+	2.69E+04	+	8.60E+04	+	5.79E+05	+	2.52E+05	+	7.74E+04
P-pocket-5	2.18E+07	+	3.94E+05	−	−	+	3.14E+05	+	2.93E+05	+	6.81E+04	+	1.05E+05
P-pocket-6	4.01E+06	+	2.66E+05	−	−	+	<100	+	2.36E+06	+	7.07E+04	+	2.42E+04
P-pocket-7	1.11E+07	+	2.60E+06	+	3.09E+05	+	<100	+	1.94E+06	+	<100	+	1.67E+05
P-pocket-8	6.67E+07	+	4.81E+05	−	−	+	3.44E+05	+	2.14E+07	+	1.06E+06	+	2.44E+05
P-pocket-9	1.88E+07	+	<100	−	−	+	1.16E+04	+	3.89E+05	+	5.76E+05	+	1.61E+04
P-pocket-10	2.29E+07	+	<100	+	1.90E+03	+	1.32E+04	+	3.15E+06	+	6.09E+05	+	3.73E+04
H-pocket-1	1.21E+06	−	−	−	−	+	<100	+	2.21E+04	+	2.01E+05	+	1.06E+04
H-pocket-2	1.04E+06	−	−	−	−	+	<100	−	−	+	3.33E+05	±	<100
H-pocket-3	2.52E+06	−	−	−	−	+	<100	−	<100	+	6.69E+03	−	−
H-pocket-4	7.39E+05	±	<100	−	−	+	2.33E+03	+	5.02E+03	+	<100	+	7.36E+03
H-pocket-5	5.88E+06	−	−	−	−	+	<100	−	−	±	<100	−	−
H-pocket-6	3.23E+06	+	1.96E+02	−	−	+	<100	+	7.14E+03	+	1.77E+02	±	<100
H-pocket-7	2.11E+06	−	−	−	−	−	−	±	<100	+	1.14E+04	−	−
H-pocket-8	4.19E+06	−	−	−	−	+	1.25E+03	+	1.61E+05	+	1.24E+05	+	3.15E+03
H-pocket-9	2.21E+05	+	1.14E+02	−	−	+	<100	−	−	+	2.52E+02	+	<100
H-pocket-10	2.63E+07	−	−	−	−	+	<100	−	−	±	<100	±	1.39E+03
P-tongue-1	1.35E+07	+	<100	+	<100	+	<100	±	4.89E+03	±	<100	±	9.36E+02
P-tongue-2	3.63E+07	+	2.79E+05	−	−	+	2.19E+05	+	8.02E+05	+	<100	+	7.10E+04
P-tongue-3	1.43E+08	+	<100	+	<100	+	2.18E+03	+	9.89E+05	+	<100	+	4.42E+04
P-tongue-4	3.47E+06	+	<100	+	<100	+	2.01E+02	±	2.74E+03	+	8.08E+03	±	1.16E+03
P-tongue-5	7.05E+06	+	1.41E+03	−	−	+	1.82E+03	+	2.74E+05	+	1.07E+05	+	1.18E+04
P-tongue-6	3.02E+07	+	<100	−	−	+	3.17E+03	+	4.78E+04	+	<100	±	6.02E+02
P-tongue-7	1.35E+08	+	<100	+	4.52E+04	+	<100	+	1.80E+05	+	<100	+	2.51E+04
P-tongue-8	1.61E+08	+	<100	+	<100	+	6.79E+04	+	2.92E+05	+	5.85E+04	+	1.10E+04
P-tongue-9	4.65E+07	+	2.30E+03	−	−	+	1.10E+05	+	1.18E+06	+	3.56E+05	+	3.24E+04
P-tongue-10	2.11E+08	+	<100	+	6.21E+03	+	4.97E+03	±	5.53E+04	+	2.10E+02	±	4.74E+03
H-tongue-1	8.83E+07	+	<100	+	<100	+	2.38E+05	+	2.97E+05	+	4.10E+02	+	2.91E+04
H-tongue-2	2.01E+08	+	<100	−	−	+	4.58E+06	±	4.27E+04	+	<100	+	1.52E+03
H-tongue-3	2.07E+06	−	−	−	−	+	9.56E+02	±	1.23E+03	+	<100	±	<100
H-tongue-4	1.01E+07	+	<100	−	−	+	8.45E+02	+	7.33E+04	±	<100	±	2.42E+03
H-tongue-5	3.47E+07	−	−	−	−	+	<100	±	6.70E+03	±	<100	±	6.64E+02
H-tongue-6	7.35E+05	+	<100	−	−	+	<100	±	9.28E+02	+	4.47E+03	±	<100
H-tongue-7	8.18E+06	−	−	−	−	+	<100	−	−	+	1.85E+04	±	<100
H-tongue-8	4.45E+06	−	−	−	−	+	1.33E+02	±	6.04E+03	+	3.51E+04	−	2.73E+02
H-tongue-9	6.67E+03	−	−	−	−	+	<100	−	−	−	−	±	<100
H-tongue-10	3.97E+07	−	−	−	−	−	−	−	−	−	−	−	−

*Crude genomic DNA samples were extracted from 10 subgingival plaque (P-pocket) and 10 tongue surface debris (P-tongue) specimens taken from patients with advanced periodontitis, as well as from 10 subgingival plaque (H-pocket) and 10 tongue surface debris (H-tongue) specimens from periodontal healthy individuals, and analyzed. *f* (+): detected, (−): not detected, (±): faintly detected.

**The real-time PCR values are expressed as theoretical cell numbers.

or absence of the target species had been established by conventional PCR.

The exact copy number of 16S rRNA operons within each cell of the numerous species of oral bacteria has not been clarified and doubling time varies among bacterial species; this represents the major limitation to the absolute determination of bacterial numbers by real-time PCR based on the 16S rRNA gene sequence (13, 25). However, it was previously reported that threshold cycles corresponding to the same amounts of genomic DNA of slow growing oral anaerobes were similar to each other (30, 40). Therefore, we utilized *P. gingivalis*, for which accurate genome size and weight are known, as the reference species to form a universal standard curve, which was then used to calculate the theoretical cell numbers of five other oral anaerobes.

The yield of the purified genomic DNA from pure cultured *P. gingivalis*, whose cell number had been calculated by colony counting, was considerably less than the theoretical weight, and the loss seemed to be unavoidable in the purification step (data not shown). Estimating cell numbers by colony counting might therefore not be suitable for a standard, though the present attempt to quantify periodontal bacteria was sufficiently reliable to compare bacterial proportions within various clinical samples. TaqMan probe/primer sets were employed for the identification and quantification of six periodontal bacteria harbored in subgingival plaque from diseased sites and on tongue surfaces of periodontitis patients by real-time PCR, and the results showed that all periodontal bacteria, except for *P. nigrescens*, were periodontitis-associated species (Table 3). These results demonstrate that such patients harbor greater amounts of periodontal pathogens in diseased subgingival sites. In addition, it was verified that the probe/primer sets reacted accurately with crude DNA samples containing target bacteria.

In the past decade, systematic bacteriology has been reorganized based on gene analysis (1, 16, 27, 58). Further, intraspecies heterogeneity among strains of *P. intermedia* was investigated and a new

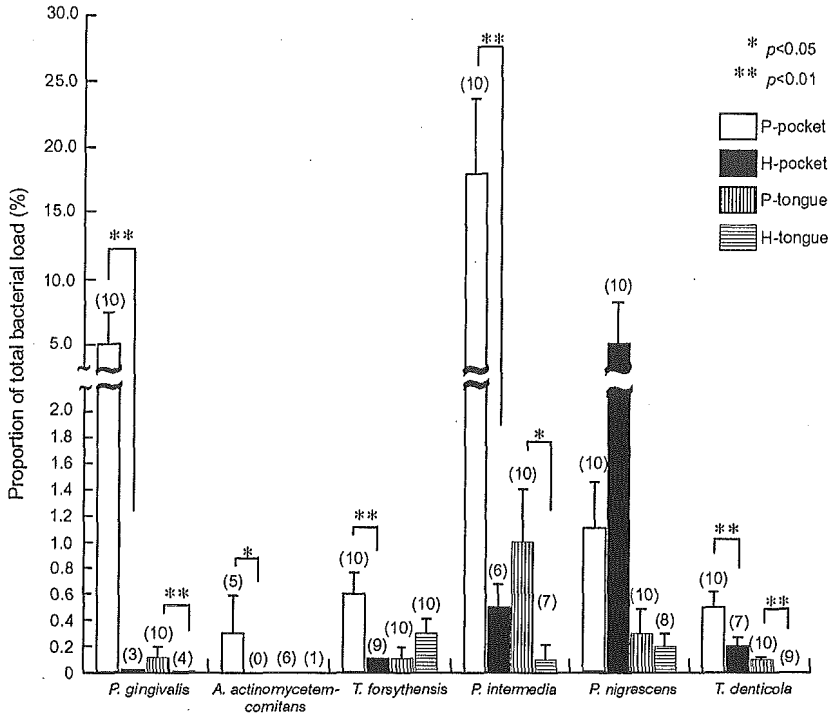

Fig. 4. Comparison of bacterial distribution in clinical specimens. The presence or absence and amount of targeted species harbored by the subjects were compared using real-time PCR. Crude genomic DNA from 10 subgingival plaque (P-pocket) and 10 tongue surface debris (P-tongue) samples taken from patients with advanced periodontitis, and 10 subgingival plaque (H-pocket) and 10 tongue surface debris (H-tongue) samples taken from periodontal healthy individuals were analyzed. Standard errors are shown as error bars. Number in parentheses: number of positive samples in which the targeted pathogen was present.

species, designated as *P. nigrescens*, was proposed (14, 16, 46). Recently, a quantitative fluorescent *in situ* hybridization (FISH) assay for differential identification of *P. intermedia* and *P. nigrescens* was reported (19); however, a quantitative real-time PCR assay for these species that is more accurate and rapid than FISH has not been described. In the present study, we used a real-time PCR analysis method to identify *P. intermedia* and *P. nigrescens* organisms according to their different variable regions of 16S rRNA gene sequences. Our quantitative assay of clinical specimens showed that *P. intermedia* was related to periodontitis, whereas *P. nigrescens* displayed no obvious tendency in association with periodontal health status (Fig. 4). This contradictory occurrence was previously reported using sensitive detection methods, though they lacked quantitative ability (9, 17, 34), and the present investigation is the first to show quantitative findings in support of those previous reports.

It is known that bacterial species occurring in tooth and tongue samples are highly associated in individuals, and that most species are more frequently detected in tongue specimens as compared to those from subgingival sites (34, 39, 51, 52). The tongue dorsum is suggested to house an organized biofilm in which anaerobic bacteria may locate and thrive, from which anaerobic locations around the teeth are seeded. This hypothesis is consistent with the concept that mucosal surfaces serve as the initial colonization site and reservoir for oral sites (6, 15, 18, 28, 35). The present findings also indicate that tongue samples are advantageous for examining prevalence. In addition, halitosis is also suggested to be related to the microbial complex on the tongue (11, 38). The present real-time PCR method may assist further investigations to understand microbial roles in that condition.

In summary, the present real-time PCR method is suitable for the detection of six putative periodontal bacteria without cross-detection of genomic products from other species. Further, results of our quantitative analysis of clinical specimens suggest that the microbial population in the oral cavity is varied depending on periodontal health status and site of sample collection. The present findings may help future microbial studies of periodontal diseases as well as those of halitosis.

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RESEARCH REPORTS
Clinical

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Association of $ALDH_2$ Genotypes and Alcohol Consumption with Periodontitis

ABSTRACT

There is little information regarding the association between alcohol consumption and periodontitis risk. We assessed whether alcohol consumption and $ALDH_2$ genotypes were associated with periodontitis. Subjects' lifestyle was examined by a self-administered questionnaire, and the percentage of pocket depths ≥ 3.5 mm was used as a periodontal parameter. $ALDH_2$ genotypes were determined with the use of a PCR/RFLP method. Multiple logistic analyses showed that alcohol consumption was significantly associated with periodontitis, and its odds ratio was 1.98. There was no significant relationship between periodontal status and $ALDH_2$ genotypes. However, $ALDH_2$*1/*2 subjects who consumed ≥ 33 g/day of alcohol had a significantly greater percentage of pocket depths ≥ 3.5 mm than those whose daily consumption was lower, while there was no significant difference in periodontal status associated with alcohol consumption in $ALDH_2$*1/*1 subjects. Our results suggest that alcohol consumption may be a risk indicator for periodontitis in $ALDH_2$*1/*2 subjects who consume larger amounts of alcohol.

KEY WORDS: alcohol consumption, periodontitis, $ALDH_2$ genotype, epidemiology

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INTRODUCTION

Alcohol consumption has been shown to increase the risk of periodontal disease, even when other lifestyle factors, including smoking, have been adjusted for (Sakki *et al.*, 1995; Shizukuishi *et al.*, 1998; Tezal *et al.*, 2001; Pitiphat *et al.*, 2003), though previous epidemiological studies have not demonstrated a definite etiologic relationship between alcohol and periodontitis risk. In humans, alcohol is first oxidized by alcohol dehydrogenase (ADH) into acetaldehyde, which is then oxidized by aldehyde dehydrogenase (ALDH) into acetate. Asian people frequently have polymorphisms in such alcohol-metabolizing enzymes as ADH_2 and $ALDH_2$, and those in $ALDH_2$ play a central role in the alcohol hypersensitivity observed in some Asians (Takeshita *et al.*, 1993). Alcohol sensitivity is highest in atypical homozygotes ($ALDH_2$*2/*2), followed by heterozygotes ($ALDH_2$*1/*2), and is lowest in typical homozygotes ($ALDH_2$*1/*1) (Enomoto *et al.*, 1991). The frequencies of $ALDH_2$*2/*2, -*1/*2, and -*1/*1 among Japanese are 6%, 38%, and 56%, respectively (Takeshita *et al.*, 1993). Since acetaldehyde, a substrate of $ALDH_2$, induces cytotoxicity, DNA damage, and an immunosuppressive effect, alcohol consumption may be more harmful for heterozygotes than for typical homozygotes (Takeshita and Morimoto, 1996). Therefore, this genotype difference in individuals may also affect the severity of periodontal disease. The purpose of this study was to assess the association of $ALDH_2$ genotypes and alcohol consumption with periodontitis.

SUBJECTS & METHODS

Study Population

In total, 453 Japanese factory workers employed at a manufacturing company in Osaka were available for study in 1998, and 409 (90.3%) were surveyed *via* an oral examination and a self-administered questionnaire. Informed consent was obtained from all subjects. Three hundred seventy-two of the workers, 290 males and 82 females (aged 20 to 59 yrs), completed all items of both the examination and the questionnaire and were included as subjects in the present study. The mean (± SD) age of the subjects was 40.5 (± 11.0) yrs, and none suffered from alcoholism at the time of investigation. Permission for this study was obtained from the Ethical Committee for Clinical Research of Osaka University Graduate School of Dentistry.

Assessment of Lifestyle

Lifestyle behavior was covered by 103 items in a self-administered questionnaire and included cigarette smoking, alcohol consumption, sleeping hours, breakfast consumption, nutritional balance, working hours, physical exercise, and mental health (Kusaka *et al.*, 1992). The questions were multiple choice, with 2 to 6 possible answers for each. We also asked questions regarding 34 items of oral health behavior (*e.g.*, frequency of toothbrushing, method of brushing cervical teeth, use of an inter-dental brush) by a self-administered

questionnaire. Each answer was dichotomized as a "good" or "not good" health practice. We calculated pack-years to evaluate cigarette smoking status, and levels of smoking were separated into high (≥ 15.0) pack-years and low (< 15.0) pack-years, which were above and below, respectively, the higher 20th percentile of the distribution. In addition, we calculated body mass index (BMI) to evaluate physical factors, and the subjects were classified into 2 groups, BMI < 25 and BMI ≥ 25.

Assessment of Alcohol Consumption

Information on drinking frequency, mean amounts of alcohol consumption *per* occasion, and kinds of alcoholic beverages—including beer, *sake,* wine, whisky, *shochu* (a distilled alcoholic beverage made from wheat or sweet potatoes), and others—was obtained by a self-administered questionnaire. We calculated average daily alcohol consumption by multiplying the mean amount of alcohol consumption *per* occasion by drinking frequency. Alcohol content was estimated to be 20.0 g for a bottle of beer, 22.0 g for a cup of *sake,* 20.0 g for a glass of whisky, 50.0 g for a cup of *shochu,* and 12.0 g for a glass of wine (Japan Health Promotion and Fitness Foundation, 2001).

Determination of $ALDH_2$ and ADH_2 Genotypes

Blood samples (2-4 mL) were obtained with permission from 235 of the subjects. DNA was extracted from 100 μL of white-blood-cell-rich plasma by means of an Isoquick kit (MicroProbe, Garden Grove, CA, USA). The polymorphism on exon 12 of the $ALDH_2$ gene was determined with the use of a polymerase-chain-reaction/restriction-fragment-length polymorphism (PCR-RFLP) method (Takeshita *et al.* 1994). Briefly, the exon was amplified by 30-35 cycles of PCR (1 min at 94°C, 1 min at 50°C, and 30 sec at 72°C). One amplification primer contained a base substitution to create Ksp632I (Boehringer Mannheim, Mannheim, Germany) at 37°C for 3-6 hrs. Digested samples were separated on gels containing 3% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME, USA) and 1% regular agarose (Sigma, St. Louis, MO, USA), and were stained with ethidium bromide. The ADH_2 genotypes were determined according to a method described previously (Xu *et al.*, 1988), with PCR and *Mae*III digestion.

Assessment of Periodontitis

Two examiners conducted the clinical examinations, which included probing pocket depth (PPD) measurements, using an automated probe (Vivadent, Schaan, Liechtenstein) with a constant force of 20 g applied to all teeth present, except for third molars. Each subject was examined for PPD at 6 sites *per* tooth, and the deepest was recorded for each. The percentage of teeth with a PPD greater than 3.5 mm was assessed as a periodontal parameter. Subjects were then classified into 2 groups, based on being above or below the upper 20th percentile of the percentage, as periodontitis or non-periodontitis, respectively. Calibrated examiners performed the periodontal examinations. The kappa value for PPD between the two examiners was 0.76, when a PPD of 3.5 mm was used as the cut-off point. The examiners were blind to subjects' alcohol consumption status.

Statistical Analysis

Data were analyzed by means of the SPSS statistical package (SPSS Inc., Chicago, IL, USA). The associations between periodontitis and surveyed lifestyle variables, including alcohol consumption, along with ADH_2 and $ALDH_2$ genotypes, were examined by a Mann-Whitney U test or a Kruskal-Wallis test. We used logistic regression analyses to determine which variables demonstrated a significant independent effect on periodontitis. Odds ratios and their 95% confidence intervals (CI) were also calculated. In addition, linear trends for risk were evaluated based on the mean values for each category of alcohol consumption. All reported *P* values are two-tailed, and those less than 0.05 were considered statistically significant.

RESULTS

The percentage of teeth with a PPD ≤ 3.5 mm varied from 0.0% to 100.0%, with a mean (± SD) of 33.0% (± 27.8%) and the upper 20th percentile of 60.0%. By bivariate analysis, the significant variables related to periodontitis were age, gender, BMI, smoking habit, alcohol consumption, and frequency of toothbrushing ($P < 0.05$, Table 1). The independent effects of the variables showing bivariate associations with periodontal disease were tested by multiple logistic regression analysis (Table 2). The significant variables in the model were age, BMI, smoking, and alcohol consumption, $P < 0.05$), with alcohol consumption showing an odds ratio of 1.98 (95% CI: 1.04-3.76).

To assess the effect of alcohol consumption on periodontitis, we classified the subjects into 4 groups according to the amount of daily alcohol consumption. After adjustment for age, the odds ratios were 0.92 (95% CI: 0.35-2.40) for < 22.0 g/day, 0.99 (95% CI: 0.47-2.07) for 22-32.9 g/day, and 2.77 (95% CI: 1.29-5.98) for ≥ 33.0 g/day of alcohol (*P* for trend = 0.0022), as compared with 0.0 g/day of alcohol. Although there was a significant trend (*P* for trend = 0.0251), the highest category of alcohol consumption did not show a significant odds ratio (2.04, 95% CI: 0.92-4.57) after an additional adjustment for smoking (data not shown).

The frequencies of ADH_2*1/*1, -*1/*2, and -*2/*2

Table 1. Association between Periodontal Status and Characteristics

Characteristics	N	%PPD[a] Median	%PPD[a] Mean Rank	*P* value
Age				
20-39 yrs	167	17.9	150	< 0.0001
40-59 yrs	205	35.7	206	
Gender				
Female	82	17.9	142	0.0003
Male	290	29.6	191	
Body Mass Index (BMI)				
< 25 kg/m²	295	22.2	166	< 0.0001
≥ 25 kg/m²	77	44.4	235	
Smoking status				
< 15.0 pack-years	274	21.4	160	< 0.0001
≥ 15.0 pack-years	98	42.9	238	
Alcohol consumption *per* day				
< 33 g	297	24.0	174	0.0189
≥ 33 g	75	33.4	206	
Frequency of toothbrushing *per* day				
≥ 2 times	224	22.2	169	0.0080
≤ 1 time	148	28.9	198	

[a] %PPD, percentage of probing pocket depth ≥ 3.5 mm.

were 6%, 33%, and 60%, respectively, and those of $ALDH_2$*1/*1, -*1/*2, and -*2/*2 were 55%, 39%, and 6%, respectively (Table 3). Although there was no significant difference in alcohol consumption among ADH_2 genotypes, the subjects with $ALDH_2$*1/*1 drank significantly more than those with $ALDH_2$*1/*2 and $ALDH_2$*2/*2. No significant difference in periodontal status was found between the ADH_2 and $ALDH_2$ polymorphism subjects (Table 3).

When the very few subjects with $ALDH_2$*2/*2 were excluded from the data, periodontal status in $ALDH_2$*1/*2 subjects tended to be worse than in those with the $ALDH_2$*1/*1 genotype (Table 4). $ALDH_2$*1/*2 and *1/*1 subjects were classified into those who drank less than 33 g of alcohol *per* day and those who consumed more. Among the $ALDH_2$*1/*1 genotype group (Table 4), periodontal status did not differ in alcohol consumption level, whereas among the $ALDH_2$*1/*2 genotype group (Table 4), those who drank 33 g or more of alcohol *per* day showed a significantly higher percentage of PPD ≥ 3.5 mm than those who consumed less. In addition, the results of multiple logistic regression analysis were quite different between $ALDH_2$*1/*1 and -*1/*2 genotypes. In $ALDH_2$*1/*1 subjects, there were significant odds ratios for BMI and smoking, but not with alcohol consumption. On the other hand, $ALDH_2$*1/*2 subjects showed significant odds ratios for alcohol consumption and age, even though smoking and other factors were adjusted for.

Table 2. Multiple Logistic Regression Analysis of Periodontitis According to Age, Gender, BMI, and Lifestyle-related Variables

Characteristics	N	Odds Ratio	95%CI[a]	*P*-value
Age				
20-39 yrs	167	1		
40-59 yrs	205	2.21	1.17 4.19	0.015
Gender				
Female	82	1		
Male	290	1.56	0.62 3.94	0.342
Body Mass Index (BMI)				
< 25 kg/m^2	295	1		
≥ 25 kg/m^2	77	3.02	1.63 5.57	< 0.001
Smoking status				
< 15.0 pack-years	274	1		
≥ 15.0 pack-years	98	2.40	1.28 4.51	0.007
Alcohol consumption *per* day				
< 33 g	297	1		
≥ 33 g	75	1.98	1.04 3.76	0.037
Frequency of toothbrushing *per* day				
≥ 2 times	224	1		
≤ 1 time	148	1.42	0.80 2.52	0.228

[a] CI, confidence intervals.

Table 3. Association of ADH_2 and $ALDH_2$ Genotypes with Alcohol Consumption and Periodontal Status

	N	Alcohol Consumption Average (g/day)	Alcohol Consumption Mean Rank	Periodontal Status (%PPD[a]) Median	Periodontal Status (%PPD[a]) Mean Rank
ADH_2					
*1/*1	15	33.9	85	45.5	135
*1/*2	78	26.4	72	27.5	115
*2/*2	142	28.6	83	29.6	113
P-value (Kruskal-Wallis test)			0.253		0.448
$ALDH_2$					
*1/*1	128	[c] 25.3 [b]	134	23.1	128
*1/*2	92	19.6	110	32.1	144
*2/*2	15	2.0	33	28.0	138
P-value (Kruskal-Wallis test)			< 0.0001		0.275

[a] %PPD, percentage of probing pocket depth ≥ 3.5 mm.
[b] $P = 0.0039$ (Mann-Whitney U test).
[c] $P < 0.0001$ (Mann-Whitney U test).

DISCUSSION

In the investigation, consumption of 33 g/day or more of alcohol was independently associated with periodontal status, based on the percentage of PPD ≥ 3.5 mm after adjustment for age, gender, BMI, smoking habit, and frequency of toothbrushing. We previously found that 60 g or more *per* day of alcohol intake was an independent risk indicator for periodontitis, according to modified CPI scores (Shizukuishi *et al.*, 1998). A Finnish study also reported that alcohol consumption had an independent association with periodontal health, based on the occurrence of PPD ≥ 3.0 mm (Sakki *et al.*, 1995), while the Erie County Study found a positive relationship among alcohol consumption, greater attachment loss, and gingival bleeding (Tezal *et al.*, 2001). This association was also found in a large prospective study (Pitiphat *et al.*, 2003). However, those previous reports did not elucidate the biological effect of alcohol on the risk for periodontitis, and the present is the first known study of both alcohol consumption and alcohol-metabolizing enzyme genotypes.

Researchers have reported that a J-shaped relationship with alcohol consumption was observed regarding cardiovascular disease (Coate, 1993) and bone mineral density (Holbrook and Barrett-Conner, 1993). Tezal *et al.* (2001) also suggested that the relationship between alcohol consumption and clinical attachment loss might show a J-shape. When we divided the subjects into 4 categories by alcohol consumption, the odds ratio for periodontitis risk among light drinkers (< 33 g/day of alcohol) was reduced, whereas that for heavy drinkers (≥ 33 g/day of alcohol) was increased, compared with that for non-drinkers. This effect can be plotted as a J-shape. However, when the data were adjusted by age and smoking, there was no significant odds ratio for heavy drinkers. Since our small sample size may have limited our power to detect this association, it is necessary to conduct future studies with a larger population.

Some reports have noted that $ALDH_2$ genotypes are a risk

Table 4. Association of Periodontal Status with Alcohol Consumption in $ALDH_2$ *1/*1 and $ALDH_2$ *1/*2 Genotypes by Bivariate and Multiple Logistic Regression Analyses

	$ALDH_2$	
	$ALDH_2$ *1/*1	$ALDH_2$ *1/*2
%PPD[a] (Median)	23.1	32.1
	$P = 0.1095$	
%PPD (Median) by alcohol consumption *per* day		
< 33 g	21.4	28.6
≥ 33 g	28.6	57.0
	$P = 0.0947$	$P = 0.0037$

Independent variables for multiple logistic regression analysis	$ALDH_2$ *1/*1 Odds Ratio	95%CI[b]	$ALDH_2$ *1/*2 Odds Ratio	95%CI
Age				
20-39 yrs	1		1	
40-59 yrs	2.06	0.80-5.28	4.44	1.02-19.39
Gender				
Female	1		1	
Male	3.31	0.67-16.32	1.34	0.13-13.32
Body Mass Index (BMI)				
< 25 kg/m^2	1		1	
≥ 25 kg/m^2	3.05	1.12-8.31	2.31	0.68-7.90
Smoking status				
< 15.0 pack-years	1		1	
≥ 15.0 pack-years	3.60	1.33-9.76	2.13	0.61-7.47
Alcohol consumption *per* day				
< 33 g	1		1	
≥ 33 g	1.02	0.39-2.66	5.36	1.39-20.77
Frequency of toothbrushing *per* day				
≥ 2 times	1		1	
≤ 1 time	0.98	0.41-2.36	1.52	0.47-4.84

[a] %PPD, percentage of probing pocket depth ≥ 3.5 mm.
[b] CI, confidence intervals.

factor for systemic diseases such as liver damage (Shibuya and Yoshida, 1988), alcohol-related asthma (Takada *et al.*, 1994), and cancer (Takeshita *et al.*, 2000). Although alcohol may also have direct effects on systemic diseases, acetaldehyde is generally more harmful to tissues and cells, since the substance is volatile and reacts easily with cellular components such as proteins and DNA, and induces cytotoxicity (Wickramasinghe *et al.*, 1986) and DNA damage (Fang and Vaca, 1995). Thus, such biochemical reactions may cause an adverse effect on host defense. In the present study, there was no significant association seen between periodontal status and $ALDH_2$ genotype. Although subjects with $ALDH_2$*1/*2 drank lesser amounts of alcohol daily, as compared with those with $ALDH_2$*1/*1, the former tended to show a higher periodontitis risk. Furthermore, those with $ALDH_2$*1/*2 who drank ≥ 33 g/day of alcohol showed a significant periodontitis risk, whereas heavy drinkers with $ALDH_2$*1/*1 did not. The activity in metabolizing acetaldehyde is higher in $ALDH_2$*1/*1 subjects than in $ALDH_2$*1/*2 subjects (Enomoto *et al.*, 1991). If subjects of both genotypes were to drink equally moderate amounts of alcohol, those with $ALDH_2$*1/*2 may have a higher circulating concentration of acetaldehyde than those with $ALDH_2$*1/*1 during consumption. Thus, it seems likely that acetaldehyde more strongly modifies periodontal health in subjects with the $ALDH_2$*1/*2 genotype than in subjects with $ALDH_2$*1/*1.

Since smoking is also an important risk factor for periodontitis and may be correlated with alcohol consumption, some degree of the observed association may be due to a residual complication from smoking. As for a dose-response relationship, we could not evaluate the effect of heavy drinking on the risk of periodontitis after the adjustment for smoking. However, in a multiple logistic regression analysis, there was a significant association between periodontitis and alcohol consumption in all subjects after adjustment for smoking and other factors, and the odds ratio was 1.98 (95% CI: 1.04-3.76). In particular, $ALDH_2$*1/*2 subjects showed alcohol consumption, but not smoking, as a significantly independent variable for periodontal risk in a logistic model. Collectively, alcohol consumption may be a weak risk indicator for periodontitis, but heavy drinkers with $ALDH_2$*1/*2 may have a greater risk. However, longitudinal studies on randomized sample populations will be necessary to clarify the causality between alcohol consumption and periodontitis.

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