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話題

唾液 sIgA 抗体と常在細菌叢

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〔KEYWORDS〕 sIgA，口腔常在細菌叢，口腔バイオフィルム

1. はじめに

唾液は，分泌型 IgA (secretory IgA；sIgA)，アミラーゼ，デフェンシン，ラクトフェリン，低分子ムチンなどを含む多くの抗菌物質を含んでいる．口腔では，口腔細菌がこれらの唾液中抗菌物質と戦いながら一定の常在細菌叢を形成している．近年，微生物の生き残り戦略としてバイオフィルムが注目され，多くの研究が行われるようになった．口腔においても口腔細菌が歯表面や口腔粘膜に付着して，増殖，凝集，菌体外多糖合成によりバイオフィルムの成熟が行われる(図 1)．成熟したバイオフィルムは，抗菌物質に対して抵抗性を有し，バイオフィルム内で菌体が殺されずに生きながらえるようにする．一方，抗菌物質である分泌型 IgA，アミラーゼ，低分子ムチンは，歯表面のハイドロキシアパタイトに吸着し，獲得ペリクルを形成している．これらの抗菌物質は，*Streptococcus sanguinis*, *Streptococcus mitis*, *Streptococcus gordonii* が結合するレセプターと

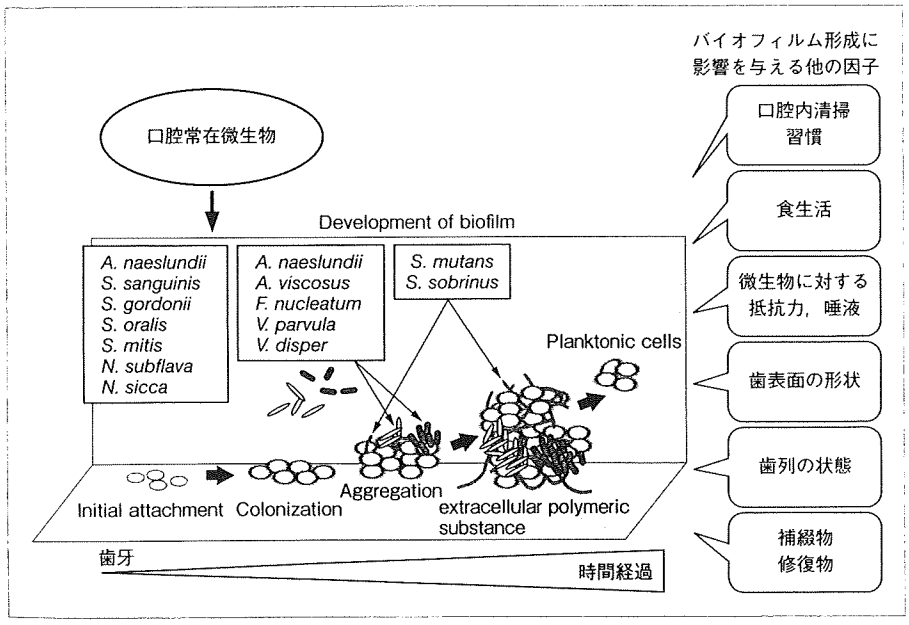

図 1　口腔バイオフィルム形成

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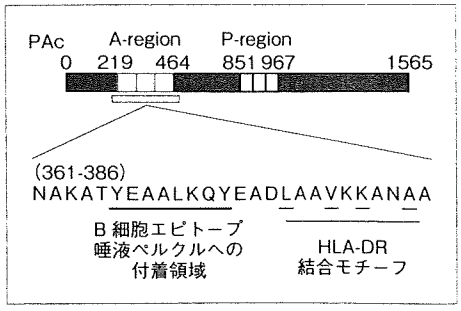

図2　*S. mutans* PAcの特徴

しても機能する．よって，獲得ペリクルとして形成されたこれらの物質にstreptococciが特異的に結合する．この現象によりstreptococciの初期付着が起こり，バイオフィルムが形成されるきっかけとなる．このような唾液成分とstreptococciの特異的な結合が起こることによって，streptococciを中心とする口腔細菌叢ができあがってくる．

streptococciの多くは，幼児期に母親などから伝播し，口腔に定着するようになる．この口腔へのstreptococciの定着により，口腔粘膜上での免疫応答や，血管に入り込んだ結果，全身免疫応答が生じ，抗streptococci抗体が誘導されるようになる．口腔には，粘膜上の免疫応答により感作を受けたリンパ球が成熟し抗原特異的なIgAを分泌，唾液腺を介してsIgAが放出されるようになる．一方，全身免疫応答では血清中に抗原特異的なIgGが分泌され，歯肉溝浸出液を介して口腔に放出されるようになる．このように唾液中には，口腔細菌に特異的に反応するIgAやIgGが存在していると考えられる．

2. 唾液抗体と結合する抗原の決定

streptococciは，菌体表面に繊毛様の表層蛋白質抗原を有している．歯表面の初期付着菌として重要な*S. gordonii*の表層蛋白質抗原SspAやSspB，齲蝕原因菌として重要な*S. mutans*のAgI/IIやPAcなどは，獲得ペリクルに存在する唾液レセプターと結合して，バイオフィルムの形成を導く[1,2]．この*S. mutans*の表層蛋白質を利用した齲蝕予防ワクチン研究が世界的に行われるようになった[3]．

筆者らも，PAcを利用したワクチンを作製することを目標に研究を行い，まずはPAcの中で唾液レセプターと結合する部分を見つける研究を行った．この部分を利用してワクチンを作製すれば，より効率の良いワクチン効果が期待できる．その結果，PAcのアミノ酸配列の中でアラニンの豊富な繰り返し領域(Aリピート)に唾液レセプター結合領域があることを明らかにした[4]．さらに，Aリピートの中で，N末端側から361番目のアスパラギンから377番目のロイシンまでの領域が，唾液レセプターと結合することを明らかにした(図2)[4]．この領域を利用すれば，齲蝕予防用のワクチン開発が可能となってくる．

しかし，実際にヒトにおいてこの領域を含む抗原を免疫して抗体が誘導できるかどうかわからない．ヒトのMHC(主要組織適合遺伝子複合体)により認識され，リンパ球によって抗原提示されなければヒトで抗体を誘導することができない．実際に，この領域にヒトMHC class Ⅱが結合できるアミノ酸配列がないことが明らかとなった[5]．そこで，その配列の周囲にヒトMHC class Ⅱと結合できる領域がないか検討し，その結果，377番目のロイシンから386番目のアラニンまでのアミノ配列の中に，HLA-DR(ヒトMHC class Ⅱ)の結合モチーフがあることが明らかになった(図2)[5]．この結合モチーフを含む361番目のアスパラギンから386番目のアラニンまでの領域は，ヒトにおいても抗体を誘導でき，なおかつ*S. mutans*歯表面付着領域も含むと考えられる．そこで，ヒトのリンパ球をNOD/SCIDマウスに移植して，人工合成したPAc(361-386)のペプチドをこの移植マウスに免疫して抗体が誘導できるか検討した．その結果，多くの遺伝子タイプの異なるHLA-DRを有するリンパを移植したマウスにおいて，特異的な抗PAc(361-386)ペプチド抗体を誘導できることを確認した[6]．よってPAc(361-386)ペプチドは，有効なワクチン抗原となりうると考えられた．

しかし，*S. mutans*が口腔常在菌であるという点や歯磨きなどの生活習慣が齲蝕発症にかかわる点などから，本当にワクチンが齲蝕に効果的であるか疑問が残る．また齲蝕が死に至らしめない疾患であるため，ワクチンそのものの副作用を考慮すると，ワクチンの効果よりもむしろリスクのほうが上回る可能性もある．一方では，唾液中に十

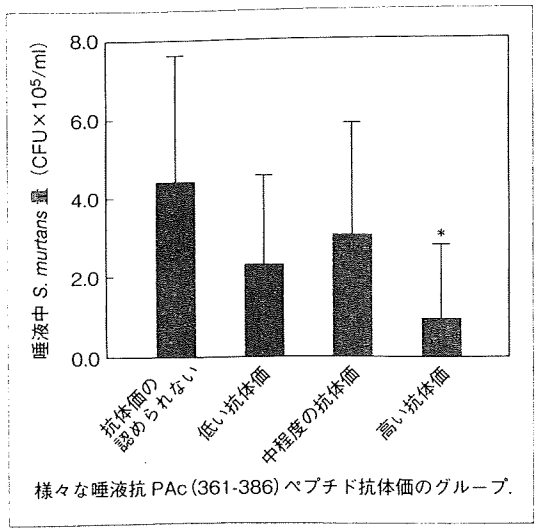

図3　抗 PAc(361-386)ペプチド　sIgA 抗体と唾液中 *S. mutans* 量との関係

*：$p<0.05$，vs 抗体価の認められないグループ.

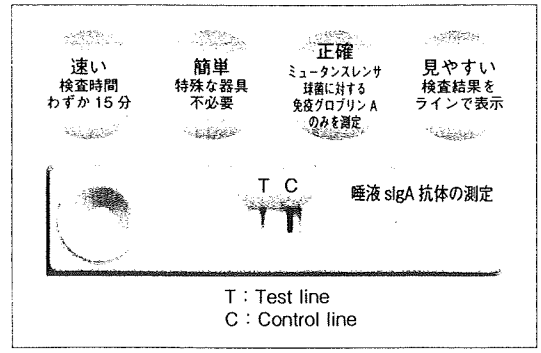

図4　唾液抗体価迅速診断検査キットの特徴

分な特異抗体を誘導する方法にも研究課題があるため，齲蝕ワクチンを実現するためには多くのハードルを越えていかなければならない.

3. 唾液 sIgA の測定

S. mutans は，母親から菌を受け継ぎ，1歳数か月を過ぎていくと口腔に定着するようになる．口腔に定着した *S. mutans* は免疫担当細胞により感作され，*S. mutans* に対する抗体が誘導されるようになる．上述の PAc に対する抗体も，成人の全身や口腔内で誘導されていることが確認された[7]．このことから，ワクチン抗原として明らかにした PAc(361-386)ペプチドに反応する抗体も同様に，*S. mutans* の口腔への感染の結果，ワクチンを免疫したのと同じように多くのヒトにおいて誘導され，抗体の効果がすでに口腔内で現れていると考えた．そこで，PAc(361-386)ペプチドに対する抗体が唾液中に存在するか検討を行った.

一般歯科医院の先生に協力をお願いして患者151人(年齢 36.6±12.6 歳)の唾液を採取し，そのサンプルを用いて ELISA(enzyme-linked immunosorbent asssy：酵素免疫測定)法で抗 PAc(361-386)ペプチド sIgA 抗体価の測定を行った．抗体価の高い唾液を有するグループ，中程度の抗体価を有するグループ，低い抗体価を有するグループ，抗体価の認められないグループの4つのグループに分け，それらのグループの唾液中 *S. mutans* 量を測定した．その結果，高い抗体価を有するグループの唾液中 *S. mutans* 量は，抗体価の認められないグループの唾液中 *S. mutans* 量よりも約 1/4 と有意に少ないことが明らかとなった(図3)[6]．これは唾液中抗 PAc(361-386)ペプチド sIgA 抗体が，*S. mutans* に作用して，*S. mutans* の歯表面への付着を阻害し，主に歯表面で増殖する *S. mutans* を減少させた結果であると考えられた．他の Streptococcus 属の菌量の目安になる総 streptococci 量は，グループ間で有意な差がなかった．この抗体は *S. mutans* のみに作用し，齲蝕になりにくい口腔環境をつくる唾液因子になると考えられる．このペプチドを用いて唾液抗体を測る検査システムを作れば，*S. mutans* に対する抵抗力がわかり，齲蝕リスクを判定できるようになる.

4. 抗体価迅速診断キットの開発

抗体価を調べる方法として ELISA があるが，ELISA を行う場合，採取した唾液サンプルを冷凍して測定するラボへ送り，約1日かけて測定し，その後結果を戻すことになる．それでは，患者さんに知らせるまで最短でも1～2日必要となる．初診来院時に短時間で測定し結果を知らせるほうが，患者さんの予防に対するモチベーションを高められる．患者さんにスムーズに予防システムを導入するためには，最低でも 30 分以内で診断できたほうがよい．そこで，短時間で抗体価を調べる方法として，金コロイドが標識された抗 sIgA 抗体をメンブレンに染み込ませ，PAc(361-

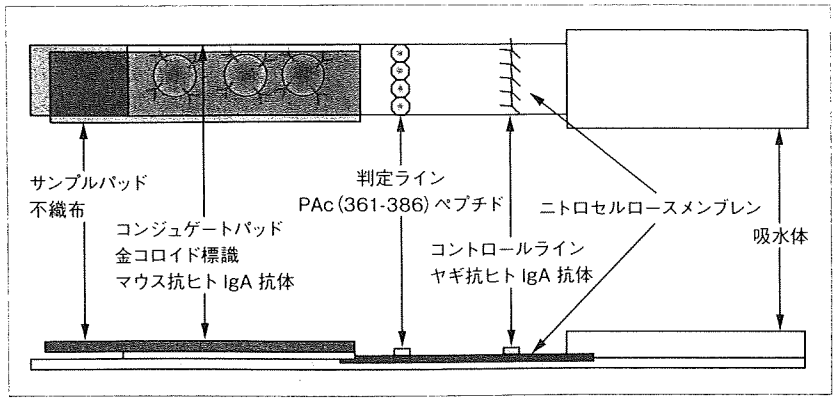

図5　唾液抗体価迅速診断検査キットの構造

386)ペプチドをテストラインに塗布するデバイスを作製した(図4)．このデバイスに唾液を染み込ませると，唾液中のsIgA抗体が金コロイド標識された抗sIgA抗体と反応し複合体が形成される(図5)．その複合体は，メンブレン中を染み込みながらPAc(361-386)ペプチドを塗布されたテストラインに到達する．複合体のうちペプチドと結合できるものがテストラインで捕らえられ，一本の目に見えるラインができあがる．その結果，抗体の有無を一目で判定することができる．唾液を染み込ませてから15分でラインが見えてくるので，短時間で齲蝕リスク判定が可能となっている．唾液をあらかじめ緩衝液で一定倍率に希釈してから使用するので，ある程度の抗体価がなければ陽性反応を示さない．この方法で高い抗体価を有する唾液を判定することができる．

5. キットを用いた齲蝕リスク判定

このキットを使用すると何がわかるか．それは上述のように唾液中の*S. mutans*の歯表面付着を阻害する抗体量，すなわち*S. mutans*に対する抵抗力である．ヒトの抗体誘導は，MHCにより規定されている．MHCに個人差があるように，PAc(361-386)ペプチドに対するsIgA抗体の誘導能力も個人により異なってくる．183人の77歳高齢者を対象にキットを用いた抗体の有無と1年後の齲蝕発症傾向を調べた．このペプチド抗体を有するヒトは年齢が高くなるとともに減少傾向にあった[6]．77歳高齢者の場合，125人が陰性，58人が陽性であった．この陰性グループの1年後のDFT(1人平均齲蝕経験歯数)の増加数は1.17±1.90であったのに対して，陽性グループの1年後のDFTの増加数は0.55±0.94で有意($p=0.017$)に減少していた．抗体が*S. mutans*へ作用して，歯表面への付着を抑えた結果，*S. mutans*量が減少し，新たな齲蝕発症が抑制されたことが考えられる．

6. まとめ

口腔常在菌叢は，そのバイオフィルム形成と口腔常在菌に対する唾液sIgAを含む抗菌物質に影響を受けながら一定のバランスのもとに維持されている．そのバランスを崩していくのは，年齢，生活習慣，全身疾患，免疫応答などの様々な因子がかかわってくる．そのバランスの崩れは，齲蝕や歯周病などの口腔疾患の発症にも繋がってくると考えられる．唾液sIgAを短時間で測定できるキットは，口腔細菌のバランスの崩れや齲蝕リスクを判定できる臨床的に有用な方法である．

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Original Article

Reduction of Gingival Bleeding by Professional Toothbrushing Compared to One-stage Full-mouth Disinfection

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Abstract

A foundation for effective periodontal treatment is the removal of subgingival plaque, and this may be carried out using the one-stage full-mouth disinfection method. In addition, the host response should be considered in periodontitis. In this study, gingival bleeding by professional toothbrushing on moderate chronic periodontitis were compared with those of one-stage full-mouth disinfection. Twelve periodontitis patients were randomly assigned to professional toothbrushing or one-stage full-mouth disinfection and received the assigned treatment daily from Day 0 to 7, and once a week from Day 7 to 28. On Day 0, 7, 14 and 28, subgingival plaque samples were obtained and clinical parameters were recorded on 4 teeth per patient. Bacterial DNAs from subgingival plaque of the teeth were analyzed using polymerase chain reaction- denaturing gradient gel electrophoresis and real-time polymerase chain reaction. Professional toothbrushing resulted in a decrease in the number of periodontal pathogens that followed the decrease in bleeding on probing. Moreover, professional toothbrushing improved the clinical parameters as effectively as one-stage full-mouth disinfection. The treatment time for professional toothbrushing was about 1/4 of that for one-stage full-mouth disinfection. These findings highlight the merit of professional toothbrushing in improving gingival bleeding and reducing the proportion of periodontal pathogens in the subgingival plaque.

Key words: Periodontitis, professional toothbrushing, one-stage full-mouth disinfection, real-time polymerase chain reaction, mechanical stimulation

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Introduction

Some 40 years ago, it was demonstrated that cessation of toothbrushing resulted in deposition of plaque followed by gingivitis; with resumption of toothbrushing, plaque was removed and inflammation resolved (L e *et al.* 1965). Results of this experimental gingivitis study confirmed the central etiological role of supragingival plaque in gingivitis. In the case of periodontal disease, subgingival plaque is considered to be the principal source of pathogenic organisms, and treatment of periodontal disease is centered around its removal, most effectively performed using the one-stage full-mouth disinfection method (Quirynen *et al.* 1995). Toothbrushing is also believed to be an effective means of helping in the treatment, as well as prevention, of periodontal disease, even though access to subgingival plaque is limited.

A growing body of evidence suggests that

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mechanical stimulation of the gingiva by toothbrushing promotes local host defense mechanisms. Toothbrushing is more effective than plaque removal with a curette in reducing inflammatory cell infiltration and increasing the number of collagen-synthesizing gingival fibroblasts (Horiuchi *et al.* 2002). Brushing twice a day reduces gingival inflammation and favorable clinical effects appear earlier than with brushing once a day (Yamamoto *et al.* 2004). Toothbrushing every other day maintains gingival health, although the amount of accumulated plaque is theoretically sufficient enough to induce gingivitis (Lang *et al.* 1973). These results suggest that the effect of toothbrushing on gingivitis is not only due to plaque removal but also stimulation of the host defense mechanism.

Toothbrushing should cover all the gingiva around a tooth, as the effect of mechanical stimulation on gingival cell activation is limited to the brushed area (Sakamoto *et al.* 2003). Self toothbrushing may not be technically enough to stimulate all the gingiva, and professional toothbrushing is recommended in periodontal diseases.

The effects of professionally delivered meticulous supragingival plaque control on clinical symptoms of periodontitis and composition of subgingival microbiota have been shown (Smulow *et al.* 1983, Dahlén *et al.* 1992, Katsanoulas *et al.* 1992, McNabb *et al.* 1992, Al-Yahfoufi *et al.* 1995, Hellstr m *et al.* 1996). In these studies, supragingival scaling and polishing were performed. No information is available regarding the clinical and microbiological effects of professional toothbrushing. Here, we performed a single-blind randomized clinical study to compare the effects of professional toothbrushing and one-stage full-mouth disinfection on the clinical parameters and subgingival microbiota in patients with moderate chronic periodontitis.

Materials and methods

Patient selection and experimental design

Twelve periodontitis patients (mean age 56 years, range 41-74 years) with at least 20 teeth and at least 4 teeth with a probing pocket depth of 5 mm or more were randomly assigned to the toothbrushing or one-stage full-mouth disinfection group. Randomization was performed using a table of random numbers. Individuals were excluded if they were pregnant, had received periodontal therapy, antibiotics or an antimicrobial product in the previous 3 months, or if they had any systemic condition which may affect the progression or treatment of periodontitis. The study protocol was approved by the Ethical Committee of the Okayama University Dental School, and subjects provided written informed consent for study participation.

Treatment in the toothbrushing group consisted of professional toothbrushing using the Toothpick Method (Morita *et al.* 1998) once daily from Days 0 to 7 and on Days 14 and 21. No oral hygiene instruction was provided. In the one-stage full-mouth disinfection group, removal of supra- and subgingival plaque and calculus was performed within the first 24 hrs (Quirynen *et al.* 1995), and supra- and subgingival plaque removal was subsequently performed from Days 3 to 7 and on Days 14 and 21. Subgingival irrigation with 0.04% chlorhexidine, brushing of the dorsum of the tongue and rinsing with 0.04% chlorhexidine were also performed in the one-stage full-mouth disinfection group. Both treatment groups maintained self-performed oral hygiene measures between visits.

Sample collection and DNA extraction

In each patient, 4 teeth with a probing pocket depth of 5 mm or more at baseline were selected as the representative teeth. Microbiological sampling was performed on Days 0, 7, 14, and 28. After removal of supragingival plaque, the sampling sites of the representative teeth were isolated with sterile cotton roll, and then air-dried. The subgingival plaque sample was removed from each pocket using a sterile curette (Paster *et al.* 2001). The sample was immersed in 1.0 ml phosphate-buffered saline (pH 7.2) (Invitrogen Co., Grand Island, NY, USA), mixed for 30 sec, and centrifuged at 12,000 g for 20 min to remove the supernatant. The bacterial DNAs were extracted from cultivated strains and clinical plaque samples using a kit (InstaGene¤ Matrix, Bio-Rad Lab., Hercules, CA, USA) and according to the manufacturer s instructions. The extracted DNAs were used in polymerase chain reaction (PCR) - denaturing gradient gel electrophoresis (DGGE) and real-time PCR analyses.

PCR-DGGE analysis

An approximately 585-bp 16S rDNA fragment corresponding to nucleotide positions from 341 to 926 in the sequence of *Escherichia coli* was amplified with two conserved primers. A forward primer, with a 40-bp GC-rich sequence added to the 5 -end (5 -CGCCCGCCGCGCCCCGCGCCCGT C C C G C C G C C C C C G C C C G - C C T A CGGGAGGCAGCAG-3), and reverse primer (5 -

Table 1. *Primers used for real-time PCR*

Targets	Forward Reverse	Sequence of the primer (5 -3)
Aggregatibacter actinomycetemcomitans[a]	F	CTTACCTACTCTTGACATCCGAA
	R	ATGCAGCACCTGTCTCAAAGC
Porphyromonas gingivalis[a]	F	CTTGACTTCAGTGGCGGCAG
	R	AGGGAAGACGGTTTTCACCA
Prevotella intermedia[a]	F	AATACCCGATGTTGTCCACA
	R	TTAGCCGGTCCTTATTCGAA
Tannerella forsythia[b]	F	GCGTATGTAACCTGCCCGCA
	R	TGCTTCAGTGTCAGTTATACCT
Treponema denticola[b]	F	TAATACCGAATGTGCTCATTTACAT
	R	CTGCCATATCTCTATGTCATTGCTCTT
Total bacteria[a]	F	GTGSTGCAYGGYTGTCGTCA
	R	ACGTCRTCCMCACCTTCCTC

[a] Designed according to published methods (Maeda *et al.* 2003).
[b] Designed originally for this study.

CCGTCAATTCCTTTRAGTTT-3) were prepared for the PCR. Amplification was performed using a kit (EX-Taq¤, TaKaRa Shuzo Ltd., Otsu, Japan) in 50- l reactions. The thermocycling program was performed as previously described (Fujimoto *et al.* 2003). Prior to DGGE analysis, the PCR products were visualized by electrophoresis in a 2% agarose gel to confirm the 16S rDNA amplification.

A DCode Universal Mutation Detection System (Bio-Rad Lab.) was used for sequence-specific separation of the PCR products (Muyzer & Smalla 1998). The PCR products were separated on a 6% (w/v) polyacrylamide gel containing a linear gradient ranging from 20 to 50% denaturant. The gel was run for 5.5 hrs at 200V in 0.5 x Tris-acetate-EDTA (TAE) buffer (1 x TAE buffer contains to 0.04 mol/l Tris base, 0.02 mol/l acetic acid and 1.0 mmol/l EDTA, pH 8.5) at a constant temperature of 60ßC. After staining with ethidium bromide, the gel was viewed under UV transillumination.

Partial 16S rDNA gene sequencing of excised denaturing gradient gel bands

The dominant 16S rDNA bands on the denaturing gradient gel were excised, purified, and re-amplified using PCR with the same conditions described above. The re-amplified DNA fragments were cloned into a vector (TOPO TA Cloning kit¤, Invitrogen, Carlsbad, CA, USA), and the insert DNAs were sequenced using a kit (ABI Prism BigDye¤ terminator v1.1 cycle sequencing ready reaction kit, Perkin Elmer, Foster City, CA, USA) and an automated DNA sequencer (ABI PRISM 3100, Perkin Elmer). The sequence data were subjected to the BLAST sequence homology search program at GenBank and analyzed. The bacterial species were identified at similarity values above 99%.

Bacterial strains and primers for real-time PCR

The bacterial strains used for real-time PCR were *Aggregatibacter actinomycetemcomitans* ATCC 43718 (Y4), *E. coli* K-12, *Porphyromonas gingivalis* ATCC 33277^T, *Prevotella intermedia* ATCC 25611^T, *Tannerella forsythia* ATCC 43037^T, and *Treponema denticola* ATCC 35405^T. Table 1 shows the sequences of primers and probes, designed using software (Primer Express, version 1.0, PE Applied Biosystems, Foster City, CA, USA; OLIGO, version 4.0, Molecular Biology Insights, Cascade, CO, USA) (Maeda *et al.* 2003). For the identification of bacterial species, primers were designed from the species-specific region on the 16S rDNA, whereas the conserved sequences were used for amplification of all bacteria present.

Real-time PCR analysis

The real-time PCR reactions were performed using the GeneAmp¤ 5700 Sequence Detection System (PE Applied Biosystems). The reaction mixture (25 l) contained 13 1 2 x double-stranded DNA-binding reporter fluorescent dye solution (SYBR¤ Green PCR Master Mix, PE Applied Biosystems), 1 l of forward and reverse primer, 2.5 l of extracted DNA and sterile distilled molecular biology grade water to adjust the volume and brought to thermocycling program (Maeda *et al.* 2003). The specificity of the amplification products was assayed with dissociation curves, constructed in the range of 60℃ to 90℃. All data were analyzed

Table 2. *Clinical findings over time by treatment group*

	Treatment group[a]	Day 0	Day 7	Day 14	Day 28
Gingival index	PT	1.9 – 0.5[b]	0.6 – 0.6[d]	0.2 – 0.4[d]	0.1 – 0.3[d]
	FDis	1.5 – 0.5 [g]	0.7 – 0.6[d]	0.4 – 0.5[d]	0.2 – 0.4[d]
Probing pocket depth (mm)	PT	6.3 – 1.5	5.2 – 1.5[d]	4.5 – 1.5[d]	4.4 – 1.5[d]
	FDis	5.5 – 0.7 [g]	4.7 – 1.1[c]	4.3 – 1.2[d]	3.7 – 1.0[d]
Probing attachment level (mm)	PT	6.5 – 1.5	5.3 – 1.6[d]	4.7 – 1.6[d]	4.6 – 1.8[d]
	FDis	5.8 – 1.0 [g]	4.9 – 1.2[c]	4.6 – 1.4[c]	4.0 – 1.4[d]
Presence of bleeding	PT	21	5[f]	3[f]	2[f]
on probing (number of sites)	FDis	21	12[e] [h]	9[f] [h]	6[f]
Plaque index	PT	2.2 – 0.7	0.6 – 0.5[d]	0.5 – 0.7[d]	0.5 – 0.6[d]
	FDis	2.3 – 1.5	0.9 – 1.1[d]	0.8 – 0.8[d]	0.4 – 0.5[d]

[a] PT = Professional toothbrushing, FDis = One-stage full-mouth disinfection.
[b] Data are expressed as mean values – (SD). (n = 24 teeth)
[c] $p<0.01$, [d] $p<0.001$ Wilcoxon signed-ranks test, compared to day 0.
[e] $p<0.01$, [f] $p<0.001$ Chi-square test, compared to day 0.
[g] $p<0.05$ Mann-Whitney U-test, compared between the two groups.
[h] $p<0.05$ Chi-square test, compared between the two groups.

using GeneAmp® 5700 SDS software (PE Applied Biosystems).

Clinical examination

At baseline (Day 0), and on Days 7, 14 and 28, the following clinical parameters were measured on the representative teeth by an examiner blinded to the treatment assignment: probing pocket depth, probing attachment level and bleeding on probing of the deepest pocket. The gingival index (L e & Silness 1963) and a modification of the Quigley-Hein plaque index (Turesky *et al.* 1970) were used to record gingivitis and plaque levels, respectively.

Blinding

Each patient was randomly assigned to one of the two treatment modalities by one researcher (JS). All other contributors to the study were blinded to the generation and implementation of the treatment assignment. The method of randomization was not revealed until all data had been analyzed. The clinical examiner (TY), the therapist (NT), microbiologists (BC, MT, SK, and KF) and the statistician (TT) were blinded to the treatment group.

Statistical analysis

Chi-square test and Wilcoxon signed-ranks test were performed using a statistical package (11.5 J for Windows, SPSS Japan, Tokyo, Japan) with the representative tooth as the unit of analysis.

Results

Bleeding on probing was less common in the professional toothbrushing group than in the one-stage full-mouth disinfection group on Days 7 and 14. There was significant clinical improvement from Day 0 to 7 in both the professional toothbrushing and one-stage full-mouth disinfection groups (Table 2).

As observed by PCR-DGGE, detection frequencies of anaerobic bacteria including *P. gingivalis*, *P. intermedia* and *T. forsythia* were reduced in both groups over time (Table 3). Detection frequencies of aerobic bacteria including *Neisseria mucosa*, *Rhizobium* JEYF14 and *Rothia dentocariosa* were increased. Real-time PCR (Fig. 1) showed that the percentages of some bacterial species including *P. gingivalis*, *P. intermedia*, *T. forsythia* and *T. denticola* decreased in both treatment groups over time.

The average (standard deviation) treatment time per day for the professional toothbrushing group was 15.7 min (2.7 min). The time required for one-stage full-mouth disinfection on the first two visits and subsequent visits was 90.3 min (4.5 min) and 29.6 min (1.9 min), respectively.

Discussion

The results of this study demonstrate that professional toothbrushing using the Toothpick method was more effective in reducing gingival bleeding than the one-stage full-mouth disinfection method. Gingival bleeding is an outer hemorrhage, which is caused by ulceration in periodontal pockets and increased capillary permeability of the gingiva. Mechanical stimulation with toothbrushing enhances proliferation of gingival epithelium and endothelial cells (Horiuchi *et al.* 2002) and thus can reduce

Table 3. *Number of teeth with each bacterial species at each time point according to PCR-DGGE*

	Professional toothbrushing (Day)				One-stage full-mouth disinfection (Day)			
	0	7	14	28	0	7	14	28
Aerobic bacteria								
Neisseria elongata	0[a]	0	0	0	0	3	1	2
Neisseria mucosa	2	6	7	8	0	11	8	9
Neisseria flava	0	0	0	0	0	5	5	4
Pedobacter sp. oral clone AV100	1	1	1	1	1	2	1	4
Rhizobium sp. JEYF14	0	0	2	4	0	2	3	4
Rothia dentocariosa	0	5	3	4	0	4	2	3
Anaerobic bacteria								
Aggregatibacter actinomycetemcomitans	2	2	4	4	0	0	0	0
Actinomyces naeslundii	2	3	2	3	0	2	5	7
Actinomyces odontolyticus	0	0	0	0	0	0	0	3
Corynebacterium durum	0	0	0	0	0	0	1	3
Corynebacterium matruchotii	1	1	3	3	1	2	6	10
Corynebacterium sp. oral clone AK153	0	0	0	0	0	1	1	2
Deferribacteres sp. oral clone D084	1	2	4	2	2	4	0	1
Deferribacteres sp. oral clone W090	0	0	1	3	0	2	4	1
Dialister pneumosintes	2	1	1	0	1	2	2	0
Eubacterium saphenum	0	0	0	0	3	1	2	1
Fusobacterium naviforme	0	0	0	0	2	0	1	1
Fusobacterium nucleatum	2	1	1	2	2	2	3	2
Haemophilus parainfluenzae	0	0	0	0	2	2	0	0
Lautropia mirabilis	10	10	9	4	6	10	8	9
Lautropia mirabilis	10[a]	10	9	4	6	10	8	9
Porphyromonas endodontalis	2	2	2	1	5	4	3	2
Porphyromonas gingivalis	23	9	9	7	24	9	14	8
Porphyromonas sp. oral clone BS077	3	4	6	3	0	0	0	0
Prevotella dentalis	0	0	0	0	2	2	0	0
Prevotella intermedia	7	4	2	0	4	3	0	2
Prevotella loescheii	4	2	2	2	0	0	0	0
Prevotella melaninogenica	3	1	2	1	0	0	0	0
Prevotella nigrescens	0	0	0	0	3	2	0	0
Prevotella odontolyticus	2	4	3	1	0	0	0	0
Prevotella oralis	1	0	0	0	4	3	3	4
Prevotella oris	4	2	1	3	5	5	1	1
Tannerella forsythia	8	8	3	1	3	2	1	0
Treponema denticola	3	2	0	0	0	0	0	0
Veillonella parvula	1	4	3	1	0	2	0	2

[a] Total number of teeth examined was 24.

gingival bleeding. These effects are observed when toothbrushing is performed with optimum force and duration in a limited area (Tomofuji *et al.* 2002, Sakamoto *et al.* 2003, Tomofuji *et al.* 2003). The Toothpick method (Morita *et al.* 1998), in which the bristles are inserted into interdental areas, was effective in reducing the gingival bleeding.

It seems unlikely that toothbrushing was able to directly remove subgingival plaque since the depth of the periodontal pockets was 5 mm or more and the filaments of the toothbrush were unlikely to reach that far into the pocket. The beneficial effects of professional toothbrushing are more likely due, in part at least, to supragingival plaque removal influencing the composition of subgingival microbiota. Some Gram-positive bacteria in supragingival plaque have been shown to serve as attachment sites for *P. gingivalis* and other gram-negative bacteria (Turesky *et al.* 1970). Moreover, bacteria in supragingival plaque produce compounds which are essential growth factors for subgingival bacteria (Slots & Gibbons 1978). Supragingival plaque removal by professional toothbrushing may also have disrupted the equilibrium of the subgingival flora by altering the supragingival environment.

The findings of this study are not in agreement with those of other studies which showed

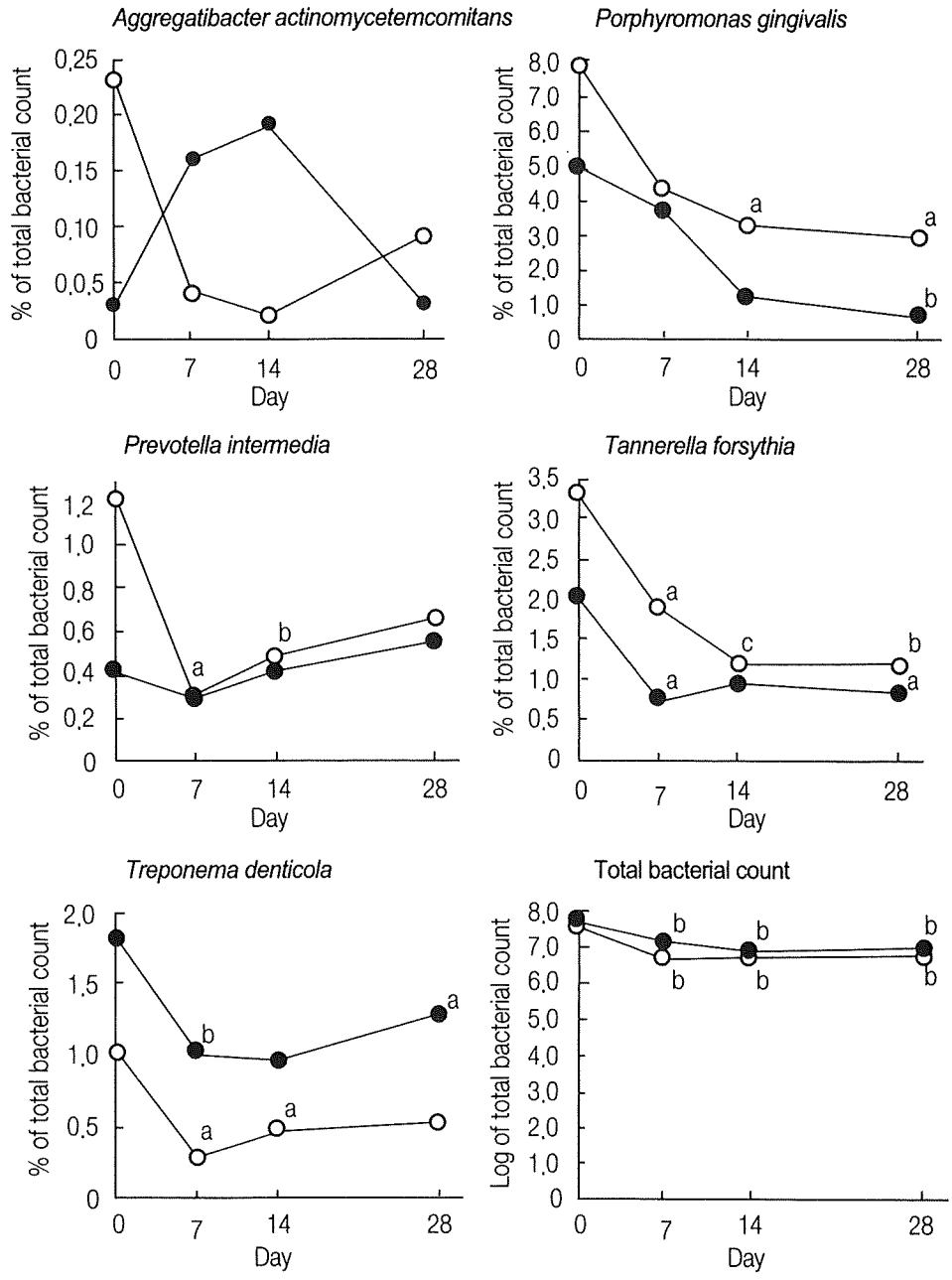

Figure 1. *Microbiological results for the professional toothbrushing group (open circle) and one-stage full-mouth disinfection group (closed circle) based on real-time PCR. Data are expressed as mean values (n=24 teeth). Data for each bacterial species are expressed as a mean percentage of the total bacterial count and data for the total bacterial counts in log of the mean value.* $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ *Wilcoxon signed-ranks test, compared to Day 0.*

supragingival plaque control alone was inferior to supra- and subgingival therapy in improvement of clinical and microbiological parameters of periodontitis (Loesche 1968, Loos *et al.* 1988). In the abovementioned studies, toothbrushing was performed by the patients themselves rather than professionally. The average time that patients brush at a dental clinic has been reported to be only 56.7 sec for the whole mouth (range 15 to 155 sec) (Westfelt *et al.* 1998). In the present study, professional toothbrushing using the Toothpick method was performed for about 15 min. To enhance proliferation of gingival cells and collagen synthesis, the recommended optimum duration of mechanical stimulation by toothbrushing is 10 - 20 sec per tooth surface (Emling *et al.* 1981, Tomofuji *et al.* 2002). This is certainly longer than the time patients generally spend brushing at home, suggesting that professional toothbrushing may be a more effective means to treat periodontal disease.

The change of bacterial composition in the professional toothbrushing group, which was confirmed by real-time PCR and PCR-DGGE analyses, may be ascribed to enhancement of

defence mechanisms against infection via mechanical stimulation of the gingiva. The epithelial surface of the periodontal pocket is often ulcerated (Tomofuji *et al.* 2003), and these weakened areas bleed, supporting the growth of several anaerobic periodontal pathogens such as *P. gingivalis* (Carranza & Camargo 2002), *T. forsythia* (Kesavalu *et al.* 2003) and *T. denticola* (Wyss *et al.* 1993), which require blood components in growth medium. The effects of mechanical stimulation include the promotion of cell proliferation in the junctional epithelium of the periodontal pocket (Yamamoto *et al.* 2004) and vascular endothelial cells in gingiva (Sakamoto *et al.* 2003). Accelerated repair of ulcerated epithelium by mechanical stimulation might stop the bleeding and reduce haemoglobin-required bacterial growth. This explanation was supported by the present temporal relation, where in the toothbrushing group the decrease in bleeding on probing preceded the reduction in *P. gingivalis* count (Fig. 1).

Limitations of this study include the small sample size. However, the results suggest that changes in host immune mechanism affect the ecology of subgingival microbiota. Further studies are required to confirm the findings of this study.

In conclusion, professional toothbrushing was more effective in improving gingival bleeding than one-stage full-mouth disinfection. However, there was no significant difference in the microbiological and clinical parameters of periodontitis between professional toothbrushing and one-stage full-mouth disinfection. Professional toothbrushing might thus hold an important place in periodontal treatment in the future.

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SHORT COMMUNICATION

Total bacterial counts on oral mucosa after using a commercial saliva substitute in patients undergoing hematopoietic cell transplantation

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Abstract
Purpose The commercial saliva substitute Oralbalance® has been reported to alleviate symptoms of postradiotherapy xerostomia in head and neck cancer patients. Oralbalance® may also be effective for xerostomia in patients undergoing hematopoietic cell transplantation (HCT) with high-dose chemotherapy and total-body irradiation. However, HCT patients are in a severely compromised condition, and saliva substitute must not promote infection. We reported previously that Oralbalance® has antimicrobial effects against microbial species detected during HCT in vitro. This study was performed to determine the in vivo effects of Oralbalance® on oral mucosal total bacterial counts in patients undergoing HCT.
Methods A total of 18 neutropenic patients undergoing HCT were enrolled in this study. Before and after 1 week of Oralbalance® use, bacterial samples were obtained from patients by wiping an area of ϕ1 cm on the buccal mucosa with sterilized cotton swabs. Total bacterial counts of the obtained samples were examined by quantitative polymerase chain reaction amplification of the bacterial 16S ribosomal RNA gene. As controls, bacterial samples were also obtained from ten healthy subjects, and total bacterial counts were examined.
Results No significant increase in bacterial count was observed with use of Oralbalance®. None of the patients showed bacterial counts above the range found in healthy controls after using Oralbalance®.
Conclusions In neutropenic patients undergoing HCT, Oralbalance® did not increase the total counts of oral mucosal bacteria beyond the range found in healthy controls. Oral care using Oralbalance® may alleviate the symptoms induced by hyposalivation without promoting infection.

Keywords Hematopoietic cell transplantation · Xerostomia · Saliva substitute

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Introduction

High-dose chemotherapy and total-body irradiation, which are performed as the conditioning regimen for hematopoi-

etic cell transplantation (HCT), are associated with xerostomia. Oralbalance® (Laclede, Inc., Rancho Dominguez, CA, USA), which is a commercially available saliva substitute, has been reported to alleviate the symptoms of postradiotherapy xerostomia in head and neck cancer patients [1, 2]. Therefore, this product may be effective in HCT patients. However, as these patients are in a markedly compromised condition throughout the period of HCT, saliva substitute must not promote infection.

Previously, we reported the in vitro antimicrobial effects of Oralbalance® against microbial species detected during HCT [3]. Oralbalance® does not facilitate increases in microorganisms detected in the HCT period in vitro [3]. In the present study, we determine the in vivo effects of Oralbalance® on total bacterial counts of oral mucosa in neutropenic patients undergoing HCT.

Subjects and methods

Subjects

A total of 18 neutropenic patients (neutrophil counts< 1,000/μL) undergoing allogeneic conventional (not reduced intensity regimen) HCT with a conditioning regimen composed of total-body irradiation (TBI) and high-dose chemotherapy at Okayama University Hospital (M, 12; F, 6; age, 42.9±16.2 years), who elected to use Oralbalance® to alleviate their symptoms of xerostomia, were enrolled in this study. The diseases in these patients were as follows: malignant lymphoma, nine; acute myeloid leukemia, four; acute lymphocytic leukemia, three; myelodysplastic syndromes, one; and solid tumor, one. Ten of the 18 subjects did not require antibiotics, while the remaining eight subjects needed antibiotics on at least 1 day during the examination period. Informed consent was obtained from all subjects, and the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences approved this study.

Oral managements and use of Oralbalance®

All subjects were referred to dentists, and necessary dental treatment had been completed before HCT. All subjects were taught about the self management of oral hygiene, tooth brushing after every meal and before going to bed, and oral rinsing with normal saline solution every 3 h during the day was also indicated. Nurses, dental hygienists, and dentists performed this oral management in patients with poor general condition. They used Oralbalance® from the day on which the patients felt xerostomia (all patients began use of Oralbalance® from 5 to 1 days prior to HCT, which corresponded to the period of the conditioning regimen for HCT composed of TBI and high-dose chemotherapy) at least four times per day, i.e., after every meal and before going to bed.

Total counts of bacteria on the buccal mucosa

Bacterial samples were obtained about 2 h after breakfast by wiping the buccal mucosa with sterilized cotton swabs over an area of ϕ1 cm before and after 1 week of using Oralbalance®. Samples were obtained from areas without ulcer. As controls, samples were also obtained from ten healthy members of the hospital staff (M, 5; F, 5; age, 30.5± 4.2 years). Controls did not use Oralbalance® and were only sampled once. Total bacterial counts were examined by quantitative polymerase chain reaction (PCR) amplification of the bacterial 16S ribosomal RNA gene (16S rDNA) as described previously [4] with minor modifications as follows. Cotton swab samples were suspended in 1 mL of PBS(-) (Gibco BRL, Grand Island, NY, USA). Aliquots of 500 μL from each suspension were transferred into new tubes and pelleted. Pelleted samples were resuspended in 200 μL of InstaGene matrix (Bio-Rad Laboratories, Hercules, CA, USA) to extract total bacterial DNA. Aliquots of 5 μL of extracted DNA were quantified by real-time PCR amplification of the 16S rDNA with SYBR Green. Real-time PCR and data analysis were performed using a GeneAmp® 5700 Sequence Detection System and GeneAmp 5700 SDS software (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Total bacterial counts before and after the use of Oralbalance® were compared by Wilcoxon's signed-rank test. P values were calculated using the statistical software StatFlex (Artech, Osaka, Japan).

Results

Changes in total bacterial counts on the buccal mucosa after using Oralbalance®

As shown in Fig. 1, there were no significant changes in total counts of bacteria on the buccal mucosa after use of Oralbalance®. The group without antibiotic use (Fig. 1b) tended to have higher total bacterial counts than the group treated with antibiotics (Fig. 1c), although the difference was not significant. After using Oralbalance®, none of the patients showed bacterial counts above the range found in healthy controls ($10^{4.2}$–$10^{5.6}$, n=10).

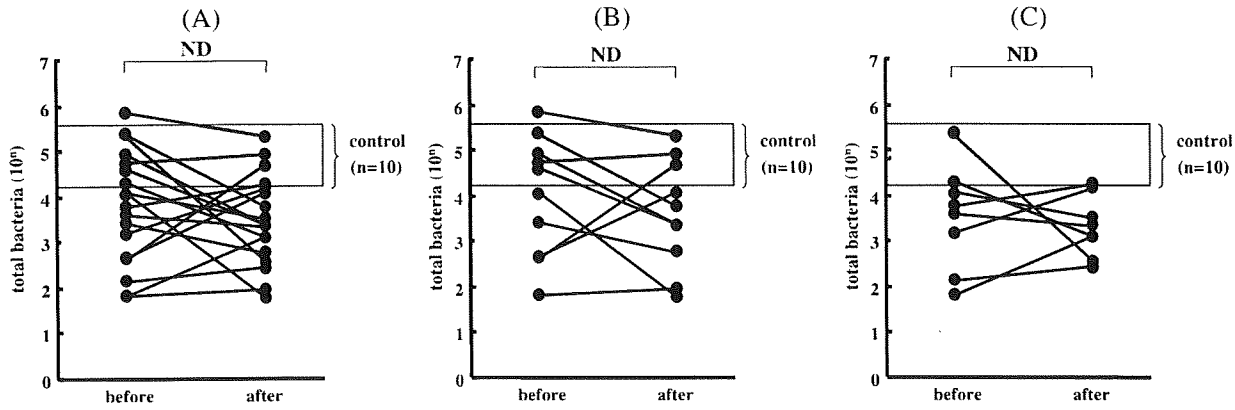

Fig. 1 Changes in total bacterial counts on the buccal mucosa with use of the mouth moisturizing gel, Oralbalance® (n=18). All patients began use of Oralbalance® from 5 to 1 days prior to HCT. "Before" using Oralbalance® samples were obtained at these points. "After" samples were obtained 1 week after starting use of Oralbalance®. **a** All subjects (n=18). **b** No antibiotics (n=10). **c** Subjects used antibiotics at least once (n=8). No significant increase in bacterial counts was observed associated with use of Oralbalance® (Wilcoxon's signed-rank test; *ND* no significant difference, $P>0.05$). Bacterial counts of healthy controls are shown in the *gray area* ($10^{4.2}$–$10^{5.6}$, n= 10). None of the patients' counts were *above* the range of those in healthy controls

Discussion

Our previous study demonstrated antimicrobial activity of Oralbalance® against the bacterial species detected during HCT in vitro and suggested that this product would not promote infection. In the present study, we performed further examination of the in vivo effects of Oralbalance® on total bacterial counts of the oral mucosa in patients undergoing HCT. No significant increases in bacterial counts were observed associated with use of Oralbalance®. It would be better to examine the differences with and without use of Oralbalance® as a case-control study. However, when this study was performed, the rumor that oral management with Oralbalance® alleviated oral pain spread among the patients in the ward. As a result, almost all patients requested oral management with Oralbalance® and a case-control study could not be performed. Therefore, we compared total bacterial counts on the oral mucosa of HCT patients with those of healthy subjects. None of the patients showed bacterial counts above the range found in healthy controls after using Oralbalance®.

In this study, quantitative PCR amplification of 16S rDNA was performed to evaluate the total counts of bacteria on the oral mucosa. Samples were obtained from areas without ulcer because of pain, and oral ulceration may influence colonization by microorganisms. Oral care was performed intensively, and no visible colonization was observed in any of the subjects during the examination period. The gene encoding the small subunit of bacterial 16S rDNA has been used frequently as a target of PCR examination because of its structural characteristics [5, 6]. The nucleotide sequences of some portions of the 16S rDNA are highly conserved through evolution [4]. The conserved sequences can provide PCR primers for amplification of 16S rDNA from all bacterial species [4]. Quantitative real-time PCR has been demonstrated to be a powerful tool for quantitative microbiological examination [4]. Therefore, we evaluated the changes in total bacterial counts on the buccal mucosa after using Oralbalance® by real-time PCR quantification of 16S rDNA. Culture and colony counting are common methods of determining bacterial counts. However, it can be difficult to culture some species of bacteria. Our results based on the bacterial DNA counts were reliable compared with culture-based methods.

The results of this study confirmed that Oralbalance® does not promote infection not only in vitro but also in vivo. Hyposalivation results in uncomfortable oral dryness, and may also increase the severity of the oral mucositis induced by chemotherapy and/or irradiation, because patients with xerostomia lose one of the most important factors involved in protecting the oral mucosa, saliva, which contains many components of the innate and acquired defense systems and not only eliminates microorganisms from the oral cavity [7, 8] but also moderates mechanical contact between the teeth and the oral mucosa. Indeed, we often see the development of ulcerative mucositis on the mucosa in contact with dry teeth clinically. Oral care using saliva substitute may alleviate the symptoms induced by hyposalivation without promoting infection. Furthermore, as Oralbalance® does not contain antibiotics, it does not contribute to the appearance of antibiotic-resistant bacteria.

In conclusion, in neutropenic patients undergoing HCT, the commercially available saliva substitute Oralbalance® did not increase the total counts of oral mucosal bacteria beyond the range found in healthy controls.

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ORIGINAL ARTICLE

Rapid detection of *mecA* and *spa* by the loop-mediated isothermal amplification (LAMP) method

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Keywords
loop-mediated isothermal amplification, *mecA*, Methicillin-resistant *Staphylococcus aureus*, naked-eye inspection, *spa*.

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Abstract

Aim: To develop a detection assay for staphylococcal *mecA* and *spa* by using loop-mediated isothermal amplification (LAMP) method.
Methods and Results: *Staphylococcus aureus* and other related species were subjected to the detection of *mecA* and *spa* by both PCR and LAMP methods. The LAMP successfully amplified the genes under isothermal conditions at 64°C within 60 min, and demonstrated identical results with the conventional PCR methods. The detection limits of the LAMP for *mecA* and *spa*, by gel electrophoresis, were 10^2 and 10 cells per tube, respectively. The naked-eye inspections were possible with 10^3 and 10 cells for detection of *mecA* and *spa*, respectively. The LAMP method was then applied to sputum and dental plaque samples. The LAMP and PCR demonstrated identical results for the plaque samples, although frequency in detection of *mecA* and *spa* by the LAMP was relatively lower for the sputum samples when compared to the PCR methods.
Conclusion: Application of the LAMP enabled a rapid detection assay for *mecA* and *spa*. The assay may be applicable to clinical plaque samples.
Significance and Impact of the Study: The LAMP offers an alternative detection assay for *mecA* and *spa* with a great advantage of the rapidity.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci (MR-CoNS) including *Staphylococcus epidermidis* (MRSE) are an important cause of healthcare and community-acquired infections (Secchi *et al.* 2008; Makgotlho *et al.* 2009). Rapid identification of MRSA and MR-CoNS in hospitalized patients is essential for administration of appropriate antibiotic therapy and infection control regimens. In addition, the increasing numbers of community-acquired infections necessitate the screening of carriers, rather than simply detecting the infected patients (Cookson 1997).

It is generally accepted that oral infectious disease and oral hygiene status are associated with general health. Colonization of pathogens such as *Staphylococcus* spp. in the oral cavity acts as a reservoir for infection of systemic organs (Sumi *et al.* 2007). Oral hygiene of the functionally impaired elderly is generally poor, and the population of individuals aged 65 and older is growing rapidly in Japan and is expected to increase 135% by 2050. For these reasons, the requirement of professional oral care by dentists or dental hygienists for hospitalized patients or dependent elderly patients is expanding dramatically, and the care is known to be effective to reduce the potential pathogens in oral cavity (Yoneyama *et al.* 1999; Ishikawa *et al.* 2008). As the opportunistic infection of MRSA or MR-CoNS in these elderly or compromised hosts can be a significant cause of morbidity and mortality, dentists must pay attention to the distribution of methicillin resistance in these strains. The rapid and accurate diagnosis of these strains is sometimes difficult because of the lack of facilities, especially in visiting home-nursing patients.

For the rapid examination of methicillin resistance in *Staphylococci*, polymerase chain reaction (PCR)-based molecular techniques have been developed by targeting the *mecA*, the gene for penicillin-binding protein 2a

(Hiramatsu *et al.* 1992), and real-time PCR is now widely used for the diagnosis (Makgotlho *et al.* 2009). In general, compared to the conventional culture method, PCR can be performed in relatively rapid and simple fashion, but special regents and apparatus such as thermalcycler are needed for the method.

The loop-mediated isothermal amplification (LAMP) method was originally developed by Notomi *et al.* (2000). The LAMP reaction can be conducted under isothermal condition ranging from 60 to 65°C, and specificity is attributable to four primers that recognize six distinct sequences. Continuous amplification under isothermal condition produces an extremely large amount of target DNA within 30 to 60 min, and the method enables simple visual (naked-eye) judgment of the DNA amplification through a colour change of the reaction mixture with SYBR green I (Iwamoto *et al.* 2003). As the method requires only one type of enzyme and special apparatus is not needed, LAMP may be suitable for onsite diagnosis of methicillin resistance in isolates of home-nursing patients or bed-side diagnosis of hospitalized patients. In the current study, the LAMP method was applied to detect the *mecA* gene in both cultivated cells and clinical samples. In addition, the method was used for detection of *spa*, the gene for protein A, unique to *S. aureus* (Hallin *et al.* 2009).

Materials and methods

Bacterial strains and clinical isolates

Three MRSA strains and five methicillin-resistant *S. epidermidis* (MRSE) were isolated from patients in Okayama University Hospital. Six MRSA strains (NCTC 10 442, N 315, 85/2082, JCSC 4744, JCSC 4788 and WIS) with each type of staphylococcal cassette chromosome *mec* (SCC*mec* type-I, II, III, IVa, IVc and V; Okuma *et al.* 2002) were kindly donated by Dr T. Ito of Juntendo University. In

Table 1 Detection of *mecA* and *spa* from clinical isolates and reference strains

Bacterial strains	No. of strains tested	LAMP		PCR	
		mecA	*spa*	*mecA*	*spa*
MRSA (SCCmec* type)					
Clinical isolates† (unknown)	3	+	+	+	+
NCTC 10442 (type I)	1	+	+	+	+
N315 (type II)	1	+	+	+	+
85/2082 (type III)	1	+	+	+	+
JCSC 4744 (tyoe IVa)	1	+	+	+	+
JCSC 4788 (tyoe IVc)	1	+	+	+	+
WIS (type V)	1	+	+	+	+
MSSA					
NBRC 14462	1	−	+	−	+
NBRC 15035	1	−	+	−	+
FDA 209	1	−	+	−	+
MRSE					
Clinical isolates†	5	+	−	+	−
MSSE					
ATCC 155	1	−	−	−	−
ATCC 12228	1	−	−	−	−
ATCC 14990	1	−	−	−	−
Streptococcus salivalius JCM 5707	1	−	−	−	−
Streptococcus sanguis ATCC 10556	1	−	−	−	−
Streptococcus pyogenes IID 866	1	−	−	−	−
Streptococcus mutans ATCC 700610	1	−	−	−	−
Enterococcus faecalis NBRC 100481	1	−	−	−	−
Escherichia coli ATCC 25922	1	−	−	−	−
Aggregatibacter actinomycetemcomitans ATCC 29523	1	−	−	−	−
Porphyromonas gingivalis FDC 381	1	−	−	−	−
Fusobacterium nucleatum ATCC 25586	1	−	−	−	−

LAMP, loop-mediated isothermal amplification; MRSA, Methicillin-resistant *Staphylococcus aureus*; MSSA, Methicillin-susceptible *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus epidermidis.*; MSSE, Methicillin-susceptible *Staphylococcus epidermis*

*Staphylococcal cassette chromosome *mec*.

†Isolated in Okayama University Hospital.