に問題が残る。また、口腔内では 再石灰化能とともに 脱灰能が被検者ことに異なるという特質かあるため、再石灰化と脱灰にかかわるとの因子で、歯質のミネラル出納か影響されたのかか きわめて判定しにくいのも事実である。口腔内試験が最良とは単純には結論できないことに留意しなければならない。

ところて 歯の再石灰化は、表層の連続性を失っていない初期う蝕病巣か唾液 すなわち歯質に対して過飽和なミネラル体液に接触する という比較的単純なメカニスムで発現する。したがって 被検食品の再石灰化能を端的に評価するには 終日口腔内に接触させ 複雑な因子を介在させるよりはむしろ単純に唾液に被検物質を添加して 再石灰化のみの反応を主として観察するほうが 物質の特性を把握しやすいといえる。

そこて 初期のスクリーニングには化学的人工口腔（本研究の食品スクリーニング試験）を また プラークに作用して間接的に歯質に影響を及ぼすメカニスムである場合は 生物学的人工口腔（本研究の人工プラーク試験をまた ヒト口腔内試験の予備試験として ヒト唾液浸漬試験（ＨＳＩテスト）を 段階的に適用することか 食品の再石灰化促進性能試験として妥当と考えられる。

最終的に ヒト口腔内での試験により効果を確認することは重要てあるたたし 口腔はきわめて変化に富む環境てあることから その試験結果が必ずしも 食品の性能を高い精度で反映しているかとうかはさらに検討が必要であろう。

E 結論

人工プラークへの被検物質の供給は主としてプラーク下にある歯質に対する脱灰抑制効果の判定を可能にしており また 人工唾液と食品の交互処理は 製品状態の食品の再石灰化促進性能の有無を簡便にスクリーニングできる方法である可能性か示唆された。

F 健康危険情報 特になし

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H　知的財産権の出願登録状況　なし

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以降は雑誌/図書等に掲載された論文となりますので、
「研究成果の刊行に関する一覧表」をご参照ください。

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Oral Streptococci Exhibit Diverse Susceptibility to Human β-Defensin-2 Antimicrobial Effects of hBD-2 on Oral Streptococci

Eisaku Nishimura [1,2] Akiko Eto [1] Masatoshi Kato [2] Shuichi Hashizume [2] Susumu Imai [1] Tosiki Nisizawa [1] Nobuhiro Hanada[1]

[1]Department of Oral Health National Institute of Public Health 1 23 1 Toyama Shinjuku ku Tokyo 162 8640 Japan
[2]Research Institute Morinaga & Co Ltd 2 1 1 Shimosueyoshi Tsurumi ku Yokohama 230 8504 Japan

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Abstract. We examined the antimicrobial effects of human β defensin 2 (hBD 2) on 17 species of oral streptococci to investigate the involvement of antimicrobial peptide activity in oral microflora develop ment and the clinical use of the antimicrobial peptide for oral microflora control Oral streptococci exhibit diverse levels of susceptibility to human β defensin 2 (hBD 2) Two major cariogenic bacterial species *Streptococcus mutans* (*S mutans*) and *S sobrinus* were found to be susceptible to the peptide indicating that it is a potential therapeutic agent for preventing dental caries *S mitis* exhibited the lowest susceptibility to the peptide *S mitis* is a major indigenous bacterium in the oral microflora and our results suggest that it might possess a certain resistance mechanism against hBD 2

In the oral cavity antimicrobial peptides such as defen sin histatin and cathelicidin play a pivotal role as a first line defense against a succession of invading bacte ria [14] β defensins are small cationic antimicrobial peptides and hBD 1 3 was detected in the salivary gland gingiva tongue and buccal mucosa [4 5 11] β defensins exert their bactericidal activity by acting on the bacterial membrane and epithelial cells of the oral mucosal barrier upregulate hBD 2 and hBD 3 upon stim ulation by bacterial components or inflammatory medi ators [5 11]

More than 500 different kinds of resident bacteria form a normal flora in the oral region exposed to the action of the host defense system [6] Therefore we assumed that commensal bacteria may possess certain mechanisms of escaping the host immune system Furthermore oral microflora found in each oral region vary in composition and antimicrobial peptides could be one of the determinants of development of normal oral flora through their activities We were also inter ested in the oral application of antimicrobial peptides for clinical uses particularly for prevention of dental caries [12] Dental caries are ideal as a model for the clinical use of antimicrobial peptides in which the peptides are easily and directly delivered to the dis eased part

In this study we examined the antimicrobial effects of hBD 2 on several *Streptococcus* species This genus includes two cariogenic species *Streptococcus mutans* (*S mutans*) and *S sobrinus* and their total population comprises the largest percentage of the total population in the oral region [6]

Materials and Methods

Reagents A synthetic hBD 2 peptide was purchased from the Pep tide Institute (Osaka Japan) The activity of the peptide was con firmed previously [7] and was examined by using an *E coli* strain IFO15044 as the control in this study The medium used for the bacterial culture was from Difco (Michigan USA) Low melt low electro osmosis ultrapure agarose (Gibco BRL USA) was used after confirming its suitability for antimicrobial assay Other reagents used were purchased from Wako or Sigma unless otherwise spec ified

Bacterial strains and antimicrobial assay The bacterial strains were obtained from American Type Culture Collection (ATCC) Gifu Type Culture Collection (GTC) or Institute for Fermentation Col lection (IFO) and are listed in Table 1 To determine the antimicro bial activity of hBD 2 against various streptococcal strains we employed the radial diffusion assay because this method has been described to be sensitive and to yield reproducible results [3 9 13] Briefly bacterial cells precultured overnight were grown to log phase in trypticase soy broth (TSB) at 37 C They were harvested by

Correspondence to A Eto *email* eto@niph go jp

Susceptibilities of oral streptococcal strains and *E coli* to

MBC (µg/ml)	Strain	
<10	*E coli*	IFO 15044
	S salivarius	ATCC9757
	S gordonii	ATCC10558^T
	S sanguinis	ATCC10556^T
	S mutans	ATCC25175^T
	S sobrinus	ATCC33478^T
	S macacae	ATCC35911^T
	S ferus	ATCC33477^T
	S criceti	ATCC19642^T
	S ratti	ATCC19645^T
	S pyogenes	GTC262^T
	S constellatus	ATCC27823^T
	S intermedius	ATCC27335^T
	S oralis	GTC276^T
	S downei	ATCC33748^T
	S agaractiae	GTC1234^T
10< <100	*S anginosus*	ATCC33397^T
100<	*S mitis*	GTC495^T

MBC minimal bactericidal concentration

centrifugation washed quickly resuspended in 1% low melt agarose/10 mM sodium phosphate buffer and spread onto a culture dish placed on a level platform to obtain a uniform layer After the bacterial layer was solidified wells 3 mm in diameter were formed in the layer The concentration of the peptide in the wells was 0 1 6 3 2 6 3 12 5 25 50 100 or 200 µg/mL The resulting diameter of the clear zone surrounding the wells was measured expressed in units (0 1mm = 1U) and plotted against the logarithm of the hBD 2 concentration [9]

Results

The 17 oral streptococcal strains used in this study are listed in Table 1 *Streptococcus* species were classified into six groups based on serological typing DNA relatedness including finger printing and 16S rRNA sequences the pyogenic anginosus mitis salivarius bovis and mutans groups [8 10] We selected representative species for each group which are frequently found in the oral cavity except for the bovis group

The antimicrobial activity of hBD 2 against oral streptococci was determined by the radial diffusion assay with a peptide concentration of 0 – 200 µg/mL With the range of peptide concentrations assayed hBD 2 was found to exert a dose dependent antimicrobial activity against all strains of oral streptococci (Fig 1) However their susceptibilities to the peptide varied To compare their susceptibilities we adopted the theoretical minimal bactericidal concentration (MBC) described by Qu et al [13] which corresponds to the x intercept of the plots indicating minimal peptide concentration required to form a clear zone [13] (Table 1) The most susceptible species tested was *S gordonii* an hBD 2 concentration

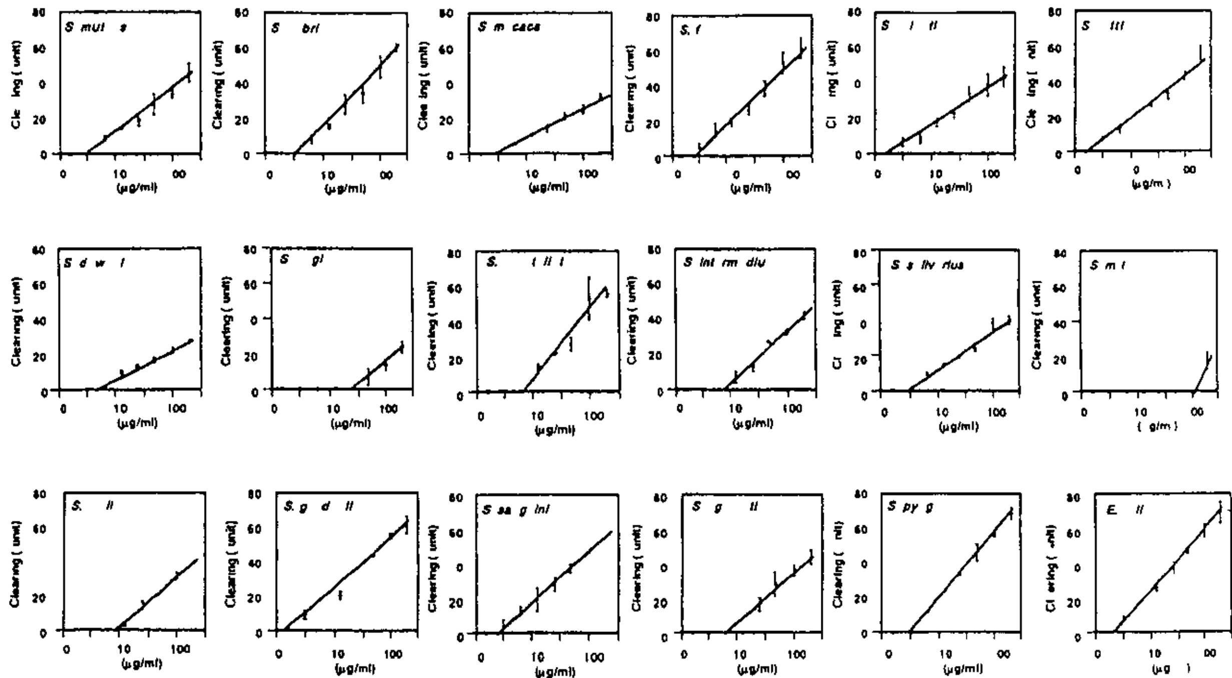

Fig 1 Dose dependent effects of hBD 2 on several species of oral streptococci The antimicrobial activity of hBD 2 was determined by the radial diffusion assay as described in Materials and Methods Each result is the mean of three experiments and error bars indicate the standard deviation

of 1 6 μg/mL was sufficient to kill this bacterium On the other hand two species *S anginosus* and *S mitis* exhibited low susceptibility to the peptide In particular an hBD 2 concentration higher than 100 μg/mL was necessary to exert its bactericidal activity against *S mitis* The low susceptibility of this species to hBD 2 is common to two strains *S mitis* GTC495T and *S mitis* ATCC6249 suggesting that this is a characteristic unique to the species (data not shown) As to its effects against pathogenic bacteria hBD 2 effectively exerted its antibacterial activity against cariogenic bacteria *S mutans* and *S sobrinus* and against inflammatory bacteria *S agalactiae* and *S pyogenes*

Discussion

Our results showed that species of the genus *Streptococcus* exhibited diverse susceptibility to hBD 2 There was no correlation between the group of strains and susceptibility Although in vivo-mimic conditioned experiments that take into consideration the nature of a biofilm might be necessary examination of the susceptibility of panels of bacterial species to antimicrobial peptides is the first step toward its clinical application [2 12] The bactericidal activity of hBD 2 against *S mutans* and *S sobrinus* suggests its potential for clinical use namely in the prevention of dental caries

Our results also revealed that *S mitis* and *S anginosus* had significantly low susceptibility to hBD 2 indicating that these bacteria may possess a certain resistance mechanism against the bactericidal action of hBD 2 This peptide is considered to act on bacterial membranes in a nonspecific electrostatic manner a certain molecule that interacts with hBD 2 before it enters the bacterial membrane could be involved in the resistance mechanism *S mitis* is one of the major bacteria in the oral indigenous microflora and it appears at the earliest stage after birth in the human oral cavity [6] Although the relationship between the host immune system and healthy normal flora has not been extensively studied [1] it is rational to consider that indigenous bacteria may possess a mechanism to overcome the host defense system leading to their coevolution

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RGD motif enhances immunogenicity and adjuvanicity of peptide antigens following intranasal immunization

Akira Yano [a] *, Atsuko Onozuka [a], Khairul Matin [a] [b], Susumu Imai [a], Nobuhiro Hanada [a], Tosiki Nisizawa [a] [c]

[a] Department of Oral Health National Institute of Public Health Toyama 1 23 1 Shinjuku ku Tokyo 163 8640 Japan
[b] Department of Prosthetics Dental College Unit Bangladesh Medical Studies and Research Institute House # 33 and 35 Road # 14/A Danmondi R/A Dhaka 1209 Bangladesh
[c] Hayashibara Biochemical Laboratories Inc 2 3 Shimoishii 1 chome Okayama 700 0907 Japan

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Abstract

The use of peptides for various aspects of medical science has been a significant advance Peptide based vaccines are promising but weak immunogenic potency is impeding the clinical application We have remarkably enhanced the immunogenicity of peptide antigens by addition of motifs that bind to cell attachment proteins such as arginine–glysine–aspartate (RGD) to the amino acid sequence The modified peptides induced antigen specific serum antibodies by intranasal immunization without adjuvants RGD an integrin binding motif was the strongest among several molecules tested in this experiment giving an average of 10 times enhancement of antibody titers when incorporated into several peptide antigens The peptides also acted as an efficient adjuvant following the intranasal immunization with protein antigens Our data support the feasibility of developing peptide vaccines and peptide adjuvants for intranasal vaccination
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Keywords Peptide vaccine Peptide adjuvant Cell attachment motif Nasal immunization

1 Introduction

A large number of researchers are investigating peptides to use as vaccines because of peptide vaccines have therapeutic properties such as safety purity stability availability and costs [1] A significant advantage is the ability to chemically synthesize peptides in any sequential amino acid arrangement However synthetic peptides vaccine development is hampered by two major problems The most critical problem is the MHC restriction [2 3] of some immune responses The impact of the MHC restriction is variable and related to heterogeneous genetic nature of humans However human T cell epitopes (HLA DR binding peptides/supermotifs) which appear to be broadly cross reactive were recently reported [4] Such broadly reactive T cell epitopes may be important components of any HIV 1 vaccine Our group is also attempting to design amino acid sequences for a single peptide that functions as a multiple T cell epitope that was recognized by several haplotypes of MHC class II genes (HLA DRs) The second difficulty is that a strong immunoadjuvant is usually needed to maximize the immune response to the weakly immunogenic peptides [1] Because of the toxicity of many adjuvants [1 5 6 7] peptide antigens that do not require co administration of adjuvants for maximum immunogenicity would be helpful for vaccine development [1 5] If immunogenicity of peptides could be enhanced by sequence alterations that allow retention of antigenic specificity they would be suitable immunogens for both routine vaccinations as well as non invasive immunization strategies such as skin and mucosal immunization [1 5]

The development of the highly immunogenic lipopeptide vaccine which facilitates the passage of lipophobic peptides through membrane barriers was a successful approach to the development of peptide vaccines [1] Other approaches for the development of peptide vaccines include multiple antigen peptide (MAP) [8] and incorporation into liposomes [9] We have designed tandem repeat type peptides that consist of two identical peptides with an amino acid spacer The tandem repeat peptides induced high antibody titers after intraperitoneal injection of mice without adjuvant [10] The production of antibodies to the new amino acid sequences generated as a result of tandem repeating could be reduced

Corresponding author Tel +81 3 5285 1278 fax +81 3 5285 1172
E mail address akiray@nih go jp (A Yano)

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significantly by di lysine spacer (KK) [11] Cathepsin B one of the important proteases for antigen processing in the context of MHC class II molecules [2] digests the di lysine amino acid sequence The reduction in antibody production to the newly generated epitope by tandem repeating of pep tides is thought to be related to the digestion of the spacer by the protease

In the present study we have used a tandem repeat pep tide antigen containing KK spacer as a model antigen for non invasive intranasal administration without adjuvant The model peptide has two identical peptide units (SmUs) separated in the center by a KK amino acid spacer The SmU a 13 mer peptide unit TYEAALKQYEADL is a min imum peptide antigen for the induction of antibodies that are cross reactive to a cell surface protein antigen (PAc) of *Streptococcus mutans* one of the causative agents of dental caries [12] The SmU induced antibodies inhibit the inter action between the PAc and salivay components [13] When the SmU alone was given to BALB/c mice intranasally as well as intraperitoneally without Freund s incomplete ad juvant (FIA) no specific serum antibodies were produced In contrast the model peptide SmU KK SmU given alone induced serum antibodies in the blood serum after either in traperitoneal or subcutaneous injections [11] In this study we have tested the benefit of adding amino acids to the model peptide to enhance its immunogenicity and improve the immunological outcome following nasal immunization

2 Materials and methods

2 1 *Peptide synthesis*

All peptides used in this study were synthesized by a step wise solid phase procedure using a Model 350 multi ple peptide synthesizer (Advanced ChemTech Louisville KY) and purified (>93% pure) by reversed phase high performance liquid chromatography on a TSK GEL column (1 by 30 cm TOSO Tokyo Japan) Peptides were verified by MALDI TOF/MS (Voyager DE^{TM}S TR work station Applied Biosystems Foster City CA) when necessary The single letter universally accepted notation for amino acids is used throughout the text

2 2 *Immunizations*

BALB/c and B10 D2 mice (both H 2^d haplotype Japan SLC Inc Shizuoka Japan) were used at 6 weeks of age to begin the immunization in all experiments Groups of five mice were immunized intranasally with 50 μg of a peptide with or without 2 μg of cholera toxin (CT) (1 μg of CT and 1 μg of CT B subunits Sigma–Aldrich Missouri MO) A micropipettor was used to gently instill 4 μl of saline solution that contained immunogen into the nasal cavities of each mouse (2 μl into each nasal orifice) Three identical booster doses were given at 2 weeks intervals

One group of mice was primed intraperitoneally with 200 μg of peptides in 200 μl phosphate buffered saline (PBS) without adjuvant Two intraperitoneal booster doses that were the same as the priming dose were given at 2 weeks intervals

One group of mice was primed intranasally with 4 μl of saline solution containing 4 μg of bovine serum albumin (BSA Sigma–Aldrich) and 1 μg of peptides or 2 μg of CT as adjuvant followed by three booster doses (same as primed) at 2 weeks intervals OVA (Ovalbumin Sigma–Aldrich) was immunized by same protocol as BSA with two booster doses

One week after the last booster dose animals were bled and serum samples were prepared from clotted blood by centrifugation and stored individually with Complete^{TM} pro tease inhibitor cocktail (Roche Diagnostics Japan Tokyo) and 0 05% (w/v) of sodium azide

2 3 *ELISA assays*

Protein antigens used for ELISA were BSA OVA and PAc Recombinant PAcs were isolated from *S mutans* TK18 as described previously [13] For the ELISA 96 well mi crotiter H plates (Sumitomo Bakelite Tokyo Japan) were coated with 2 μg per well peptide or 1 μg per well protein antigen in 100 μl of 50 mM carbonate buffer pH 9 6 and held overnight at 4 °C Plates were blocked for 1 h at 37 °C with 2% (w/v) skim milk in PBS containing 0 05% (v/v) Tween 20 (PBST) After washing with PBST 100 μl aliquots of two fold serial dilutions of mouse antiserum made in PBST with 1% skim milk were added to the wells and in cubated for 1 h at 37 °C Plates were rewashed with PBST and further incubated for 1 h at 37 °C with 100 μl of al kaline phosphatase conjugated goat anti mouse IgG (heavy and light chains) antiserum (Jackson ImmunoResearch Lab oratories Inc PA) Unbound conjugate was washed away with PBST and bound antibodies were detected by incuba tion for 1 h at 37 °C with *p* nitrophenyl phosphate (1 mg/ml) substrate The $OD_{405-690}$ was measured using a microtiter plate reader (Multiskan Bichromatic Labosystem Helsinki Finland) The ELISA antibody titer was expressed as the re ciprocal of the highest dilution giving an $OD_{405-690}$ of 0 1 unit above that of the control wells without antigen

3 Results

3 1 *Effect of cell attachment motifs*

Short protein molecules are being used as anchors for drug development Some of these molecules include arginine–glysine–aspartate (RGD) that represent integrin binding motifs and cell attachment motifs derived from cellular adhesion protein molecules such as fibronectin col lagen vitronectin fibrinogen laminin and many microbial proteins including the Tat protein of the human immun odeficiency virus (HIV) [14–16] Unmodified short length

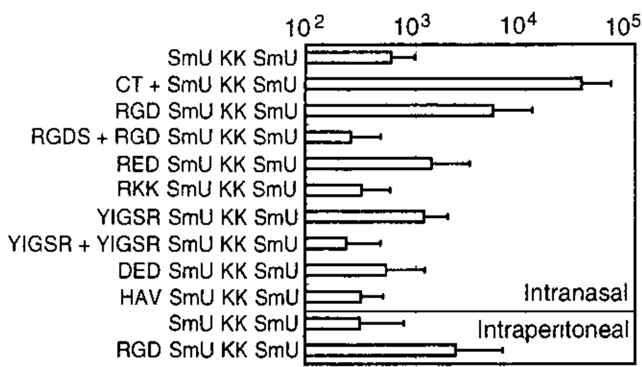

Fig 1 Anti PAc serum titres induced by intranasal or intraperitoneal immunization of peptides with addition of various binding motifs for cell attachment proteins BALB/c mice were intranasally immunized by 50 μg of each peptide either with or without 2 μg of CT followed by three subsequent booster doses at 2 weeks intervals For intranasal administration of oligopeptides 50 μg of RGDS or YIGSR was mixed with each peptide antigen solutions Two hundred micrograms of peptides were used for intraperitoneal immunization without adjuvant followed by two intraperitoneal booster doses given at 2 weeks intervals After 1 week from last booster doses serum samples were collected and anti PAc serum titres were determined by ELISA Average titers are indicated as an open box with S D bars for each group

synthetic peptides containing the RGD sequence show integrin binding activity [17] The peptides that contain the cell attachment motifs promote cell adhesion when immobilized onto surfaces of culture vessels and inhibit adhesion when incubated in solution with cells [14–16] We have added various cell attachment motifs (RGD [14 15] RED [14] LDV [14] PHSRN [18] RKK [19] DGEA [20] YIGSR [16] IKVAV [16] IRVVM [21] and RFYVVMWK [21]) to the amino terminal (N terminal) of SmU KK SmU peptides Fig 1 shows that some of these peptides significantly increased the production of specific serum antibodies after intranasal installation without adjuvant Addition of RGD and RED commonly recognized sequences for integrins to the N terminal of the SmU KK SmU enhanced the production of antibodies to PAc (cross reactive antibodies to PAc) Anti PAc titers induced by SmU KK SmU were less than 10^3 Addition of the RGD motif resulted in a titer increase to approximately 10^4 When intranasal adjuvant CT [22] was incorporated with the peptide as positive control the anti PAc titer was over 10^4 In all occasions titer of the normal serum ranged from 10 to 10^2 (data not shown) Clear but less potent enhancement was achieved with the YISGR cell attachment motif of laminin The YISGR motif does not have direct relationship with the integrin binding motifs [16] Cadherines are calcium dependent cell adhesion molecules characterized by the distinctive sequence motif that is tandemly repeated in their extracellular segments [23] HAV sequence was important sequence for chadherine binding [23 24] and DED and DRE sequences were frequently appeared in extracellular segments [24] DED DRE (both of which have amino acid sequence homology with RED) and HAV were added to the peptide there was no appreciable boost in antibody production

RGDS tetra peptide an inhibitor for RGD containing protein mediated cell adhesion [17] significantly blunted the enhancement of antibody induction by the RGD SmU KK SmU peptide Similarly enhancement of antibody induction by YIGSR motif was reduced by addition of unattached YIGSR pentamer (Fig 1) The reductions in antibody production that occurred due to the co immunization of free peptides indicate that the enhancements of the immunogenicity of newly designed peptides are related to the interactions between the peptides and their target molecules

3 2 *Importance of the RGD insertion site determination*

To determine the most effective point of insertion of the RGD for enhancement of the peptide immunogenicity several peptides were newly designed (Fig 2) To distinguish the N and carboxy (C) terminal sides of the di lysine spacer the N terminus of the SmU peptide was substituted with the OMP peptide sequence of LAVYWELLAKYLL DRVQKVA The OMP sequence has no homology with that of SmU The resulting OMP KK SmU peptide was synthesized and the RGD motif was added in the four different site of this peptide using the KK sequence as a linker The peptides to which RGD had been added induced higher antibody titers to each immunizing peptide than that to OMP KK SmU (Fig 2) This enhancement of the immunogenicity of the peptide by addition of RGD was observed in both BALB/c and B10 D2 mice that were used in this study (Figs 1 and 2) However the RGD at the C terminal side of KK linker that is OMP KK RGD SmU was less immunogenic than when RGD was at other positions on the peptide The anti immunizing peptides and the anti PAc titers induced by RGD OMP KK SmU and OMP RGD KK SmU were almost same

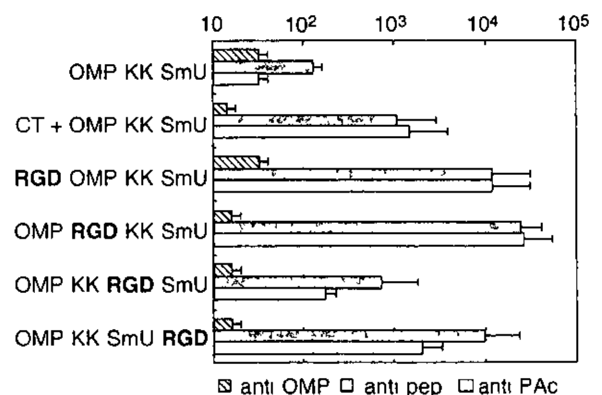

Fig 2 Effect of the position of RGD on induction of specific antibodies B10 D2 mice were immunized intranasally followed by two booster doses Serum antibody titres were determined by ELISA Average titres were indicated as hatched box (anti OMP) grey box (anti peptide) and open box (anti PAc) with S D bars for each group

3.3. Effects of RGD sequence on immunogenicity of various peptide antigens

To verify whether addition of integrin-binding motifs to peptides is a universal strategy for enhancing immunogenicity of peptide antigens, several partially substituted SmU-KK-SmU peptides were synthesized and analyzed for ability to induce antibody when used as intranasal immunogens (Fig. 3).

We used T1, OMP and OVAp, for substitution of SmU peptide. The T1 peptide (KQIINMWQAVGKAMYA) is a 16 amino acid T cell epitope of HIVIIIB gp120 that is recognized at multiple MHC loci [25]. The T1 peptide functions without species restrictions since it can be recognized by multiple mouse strains, goats, monkeys, chimpanzees and humans [26]. OVAp (OVA 323–336: ISQAVHAAHAEINE) is a 14-mer peptide antigen derived from OVA and well-studied for antibody induction in H-2^d mice [27]. In our experiments shown in Fig. 3A, OVAp was used instead of SmU as a B cell epitope for the induction of cross-reactive antibodies. Enhancement of the immunogenicity of the OMP-KK-OVAp by addition of the RGD was indicated on anti-immunizing peptide titers. The anti-OVA titers were same or higher level than anti-peptide, and anti-OMP peptide titers were low by the immunization of OMP-KK-OVAp and RGD-OMP-KK-OVAp. However, when CT was used as a immunoadjuvant, higher level of anti-OMP antibodies were induced. Fig. 3B showed the enhancement of immunogenicity of T1-KK-SmU peptide by insertion of RGD sequence at the N-terminal side of the -KK- linker. T1 peptide that was on the N-terminal side of -KK- linker was minor B cell epitope and RGD sequence enhanced the immunogenicity of the SmU peptide that was on the C-terminal side. CT also enhanced the anti-immunizing peptide titer but not the anti-SmU (anti-PAc) or anti-T1 peptide titers.

Remarkable enhancement was observed in the induction of antibodies by the peptides due to the addition of RGD or use of CT in each case, although the levels of enhancement were not the same (Figs. 1–3) and the manner of the enhancement were different between RGD addition and use of CT (Figs. 2 and 3).

3.4. Peptide antigens as peptide adjuvants for intranasal immunization

We investigated whether peptide antigens that strongly induce antibodies after intranasal instillation have adjuvant properties for intranasal immunization with BSA or OVA.

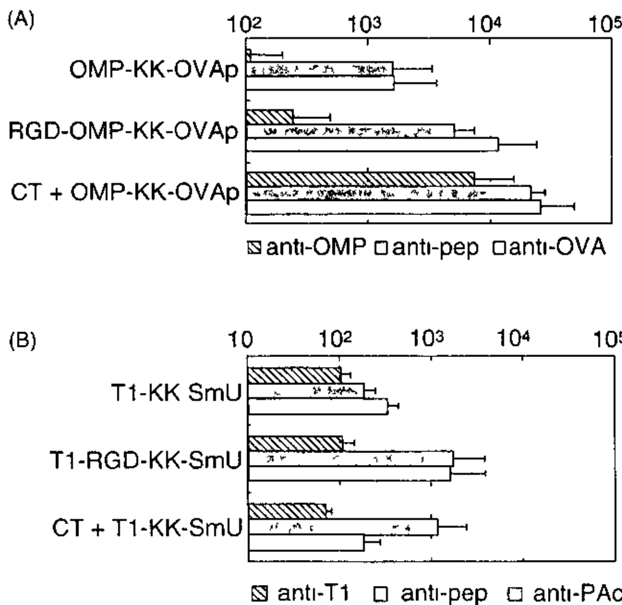

Fig. 3. Effect of RGD on immunogenicity of peptides with OMP and OVAp as substitute for SmU-KK-SmU. BALB/c mice were immunized intranasally followed by three booster doses, then specific antibody titres were determined by ELISA. Panel (A): Average titres were indicated as hatched box (anti-OMP), grey-box (anti peptide) and open box (anti OVA cross reactive antibodies to OVA in the figure) with S.D. bars of each group. Panel (B): Average titres were indicated as hatched box (anti T1), grey box (anti-peptide) and open box (anti PAc) with S.D. bars for each group.

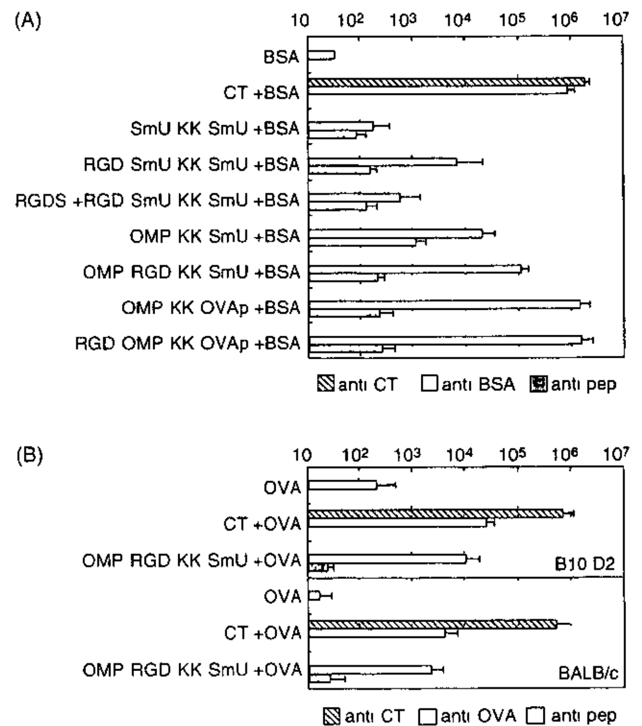

Fig. 4. Adjuvant effects of peptides on intranasal immunization with BSA and OVA. Panel (A): BALB/c mice were intranasally immunized with BSA (4 μg) with or without peptides (1 μg) or CT (2 μg) followed by three booster doses. Average titres were indicated as hatched box (anti-CT), open box (anti BSA) and grey box (anti peptide) with S.D. bars for each group. Panel (B): BALB/c and B10.D2 mice were immunized intranasally by OVA (4 μg) with peptides (1 μg) or CT (2 μg) followed by two booster doses. Average titres were indicated as hatched box (anti CT), open box (anti OVA) and grey box (anti peptide) with S.D. bars for each group.

Intranasal installation of BSA alone failed to induce serum antibodies beyond the pre-immunization levels of 10–10^2, however co-immunization of BSA and peptides significantly induced anti-BSA serum antibodies (Fig 4A) The level of anti-BSA serum antibody titres were enhanced in conjunction with the antigenicity of the peptide adjuvant These results indicate that the RGD motif added peptides, acting as adjuvants, further enhanced the induction of anti-BSA serum antibodies The adjuvant effect of peptide antigens was also demonstrated after OVA and peptide co-immunization of two strains of mice having the same H-2^d haplotype (Fig 4B) The adjuvant potency of RGD modified peptide was reduced by co-immunization with RGDS tetra-peptide (Fig 4A) A similar reduction in immunogenicity was observed with addition of RGDS to the immunization schedule with RGD-SmU-KK-SmU peptide (Fig 1) These results support a correlation of immunogenicity and adjuvanicity of peptide antigens Serum antibodies to the adjuvant peptide were only weakly induced while antibodies to BSA and OVA were induced to high titers (Fig 4) In contrast, when CT was used as a mucosal adjuvant for peptide immunization, serum antibodies to CT were induced to high titers that approached 10^6 titers (Fig 4)

4 Discussion

The RGD, RED and YIGSR cell attachment motifs used in this study, enhanced the immnogenicity of peptides The incorporation of the motifs into peptide sequences resulted in the ability to induce the specific serum antibodies using intranasal immunization and in the absence of adjuvants The effect may be related to the binding of the amino acid sequence to the cell surfaces, however further studies are necessary to understand how these motifs enhance immunogenicity There are also sequence-specific differences in the enhancing effects of the RED, DRE and DED on immunogenicity of the peptides Despite similar chemical and physical properties, only the RED motif enhanced immunogenicity of the peptide The effectiveness of the RED motif may be because is could bind specifically to the cell surface The inhibition of the immunization of RGD and YIGSR added peptide by RGDS tetramer and YIGSR pentamer also reinforced to conclusion regarding the sequence specificity of the enhancing effect Short peptides usually do not influence the effects of immunization and are well known as inhibitors of cell adhesion [14–16] Co-immunization of RGD added peptide with RGDS tetramer by same weight (about eight times by number of moles) reduced the induction of specific serum antibodies by 10%, similar reduction also observed in co-immunization of YIGSR added peptide with YIGSR pentamer These data indicate that the enhancement of immunogenicity of peptides by addition of the cell attachment motifs probably depends on the binding of the peptides to specific receptors during transport through the mucous membrane

The modification of peptide antigens with cell attachment motif sequences could produce new B cell epitopes that would induce antibodies that are diminishing the usefulness of such peptide vaccines Our studies show that the use of the -KK- linker may overcome the problem of generation of unexpected immune responses The peptides containing -KK- linker, with or without RGD motif, posses interesting immunological properties When the peptide is on the N-terminal side of -KK- linker, peptide antibody induction is weak However, positioning the peptide on the C-terminal side of -KK- linker results in strong induction of peptide antibodies Experiments using an intraperitoneal immunization schedule gave identical results (data not shown) Addition of the RGD motif on the N-terminal side of the -KK- linker seems to have further enhanced the strong induction of antibodies to the peptide on the C-terminal side of the -KK- linker According to the results in Figs 2 and 3, anti-peptide titers represented all of the antibodies induced by the peptide antigens, then they indicated that almost of all antibodies induced by the peptides were anti-SmU (anti-PAc) or anti-OVA Thus, they showed that C-terminal peptide SmU of the RGD-OMP-KK-SmU, OMP-RGD-KK-SmU and T1-RGD-KK-SmU or C-terminal peptide OVAp of the RGD-OMP-KK-OVAp were major epitopes of the immunized peptides The OMP peptide or T1 peptide are less immunogenic than SmU or OVAp (data not shown), but they are enough immunogenic when immunized with FIA However, they induced only background levels of antibodies in the N-terminal side of the -KK- linker Those data clearly showed the peptides with the RGD motif at the N-terminal side of the -KK- linker strongly induced the peptide antibodies on the C-terminal side of the -KK- linker However, only minimal levels of peptide antibodies were induced when the determinant was on the N-terminal side of the -KK- linker The -KK- linker greatly reduced the induction of the antibodies that could recognize the -KK- including regions [11]

To design a clinical vaccine, we might use this strategy to avoid the RGD generated new B cell epitopes, as well as to provide a space for another peptide with T cell epitopes Such a strategy would help to solve the MHC-restriction that currently limits the usefulness of peptide vaccines Specifically, the suggested design of peptide vaccine would be for one that contains a B cell epitope for induction of desired antibodies at the C-terminal side and a T cell epitope with the binding motifs of cell attachment molecules at the N-terminal side of the -KK- linker For examples T1 [25] or the most recently reported human T cell epitopes (HLA-DR-binding peptides) [4] could be added at the N-terminal side of the -KK- linker with RGD

Most vaccines currently in use require parenteral administration However, the use of needle and syringe vaccine administration poses significant problems in areas of the world where health needs are undeserved due to economic constraints [28] The availability of vaccines that could be administered using non-invasive methods would be a major

benefit to vaccine use Powerful adjuvants are required to attenuate enough immune response of vaccines that are delivered mucosally For example CT and *Escherichia coli* enterotoxin (LT) [7 22] are powerful mucosal adjuvants but require detoxification before human use Recently safer detoxified mutants of both CT and LT mucosal adjuvants have been produced using recombinant DNA technology [7 22] Other molecules that are immunogenic when given mucosally such as plant lectin [29] CpG oligonucleotides [30] are being studied as mucosal adjuvants The use of many of the new adjuvant candidates is limited by their immunogenicity [29] In this report we demonstrated that the nasally immunogenic peptides can also be adjuvants for BSA and OVA proteins that are given intranasally At present state the ability of the peptides as why the peptides function as nasal adjuvants is not understood However a distinct advantage was weak induction of the antibodies against the peptide adjuvants themselves as compared to currently used mucosal adjuvants A strong immune response against an adjuvant presents a clinical risk when used repeatedly Whether the peptide adjuvants described in our study can be used on a repeated basis requires study Peptides are now accepted for clinical use and have been used as therapeutic agents for angiogenesis [31] osteoporosis [32] thrombosis [33] and tumor [34] However the optimal ratio of antigens to peptide adjuvants must be adjusted carefully for each combination that will be used as vaccine

In conclusion the present study demonstrates peptide design that enhances the immunogenicity of peptide by addition of certain cell attachment motifs The designed peptides induce serum antibodies to the peptides following intranasal immunization that is administered without adjuvants The typical integrin binding motif RGD which enhances the induction of antibodies shows the most potential for intranasal immunization at this time Our results also show that the peptides can be used as efficient adjuvants for the intranasal immunization although the underlying mechanisms are not yet known It would be of interest to investigate whether our peptide vaccines and peptide adjuvants can enhance mucosal immunity Based on the promising outcome of the experiments in animals immunized with a peptide of novel design we suggest that the same design strategy might be universally employed for the development of peptide vaccines and peptide adjuvants for intranasal vaccination

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Systemic Diseases in Association with Microbial Species in Oral Biofilm from Elderly Requiring Care

H Senpuku[a] A Sogame[b] E Inoshita[c] Y Tsuha[a] H Miyazaki[d] N Hanada[e]

[a]Departments of Bacteriology National Institute of Infectious Disease, Tokyo, [b]Fukuoka and [c]Hikone,
[d]Department of Preventive Dentistry, School of Dentistry, Niigata University, Niigata, and
[e]Department of Oral Health, National Institute of Public Health, Tokyo, Japan

Key Words
Biofilm bacteria Systemic disease *Candida albicans* *Pseudomonas* spp

Abstract
Background The oral cavity is a reservoir for colonization and infection of systemic organs by pathogenic bacteria It is understood that aging, tooth eruption, hormonal changes, active disease, oral hygiene, and other factors have an influence on biofilm formation and bacterial accumulation in the oral cavity ***Objective*** To understand the influence of systemic health care on microfloral changes, we conducted epidemiological studies of nursing home residents in an attempt to elucidate the relationship between underlying systemic diseases and the isolation frequency of oral opportunistic pathogens ***Methods*** The prevalence of bacteria and fungi causing pneumonia in association with oral biofilm bacteria were determined using detection culture plates The influences of gender, age, denture-wearing status, number of teeth, and bedridden status in the patients residing in nursing homes were then analyzed ***Results*** The isolation frequency rates of *Candida albicans*, Pseudomonadaceae, *Staphylococcus* spp , and some strains of Enterobacteriaceae in plaque samples, as well as *C albicans* and *Xanthomonas maltophilia* in samples from the pharynx, were significantly higher in those requiring systemic care (mean age 83 9 years) than in those who did not require such care (mean 71 0 years) In particular, the frequencies of *Pseudomonas* spp , *C albicans*, and *Serratia marcescens* in plaque were significantly higher in those who were bedridden Furthermore, the isolation of *Pseudomonas* spp and *Klebsiella pneumoniae*, and/or *C albicans* in plaque was significantly associated with heart disease ***Conclusion*** The coexistence of *Pseudomonas* spp and *C albicans* in elderly with 10–19 teeth is a potential indicator of high risk for pneumonia and heart disease Therefore, attention to oral hygiene and professional care for removing the indicators may diminish the occurrence of systemic disease in the elderly requiring systemic care

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Accessible online at
www karger com/ger

Hidenobu Senpuku
Department of Bacteriology
National Institute of Infectious Diseases
1 23 1 Toyama Shinjuku ku Tokyo 162 (Japan)
Tel +81 3 5285 1111 Fax +81 3 5285 1172 E Mail hsenpuku@nih go jp

Introduction

In Japan, the average age is increasing rapidly while the birth rate is decreasing, and elderly people will account for approximately 25% of the population by 2025. Accordingly, the number of bedridden elderly requiring systemic care in residential and nursing homes is also increasing. In the UK, some investigators have reported that institutionalized elderly have worse oral health than those who live independently at home [1, 2]. Moreover, changes in microflora that are related to poor oral health include an increase in the prevalence of some bacteria and may also contribute to the development of pneumonia [3], as aspiration of bacteria present in oropharyngeal flora into the respiratory tract is a risk factor in elderly and compromised hosts [4], and the oral cavity is a reservoir for pathogenic bacteria recolonization that may infect systemic organs.

Oral biofilm is produced by the sequential attachment of a number of bacteria, and is dependent on both species and surface composition [5–10]. These attaching bacteria are able to accumulate on the surface [10]. However, biofilm is known to evade antimicrobial challenges involving antibiotics or host immune defenses by multiple mechanisms [11–14], and it has been shown that antimicrobial agents fail by not being able to fully penetrate it [11]. Furthermore, the bacterial community may increase in the oral environment, presenting considerable hygiene and host-defense problems in elderly people [15].

In the present study, the isolation frequencies of opportunistic pathogens from biofilm on tooth surfaces and in the pharynx were examined in the elderly requiring care. We recorded data on the prevalence of bacteria and fungi causing pneumonia in association with oral biofilm bacteria in order to determine the influence of various factors such as gender, age, denture-wearing status, number of teeth, and bedridden status in patients residing in nursing homes. Associations between opportunistic pathogens and systemic disease were also analyzed in these nursing care patients. Our results provide information on microbial frequency in oral biofilm and show the necessity of maintaining good oral hygiene during systemic health care for the elderly in residential and nursing homes.

Subjects and Methods

The study population was composed of 329 elderly (age 83.9 ± 7.5 years, 67 males and 262 females) requiring systemic care who resided in 5 different nursing homes in Fukuoka, Japan. They were placed into 4 categories: not bedridden, able to support themselves; slightly bedridden: confined to their bed to some degree; moderately bedridden, confined to their bed for a long time, and completely bedridden, unable to stand or sit. The subjects were compared to 464 controls who lived independently in their own homes and were self-supporting to determine risk factors. Both groups were compared for number, age, and sex ratio, and the results are shown in table 1.

To analyze the relationships between plaque microbial count and number of remaining teeth, we divided all subjects into 4 subgroups: those with 0, 1–9, 10–19, or 20 or more teeth in the oral cavity. This grouping by number of teeth has been referred to in previous reports [16, 17]. The typical number of teeth in 80-year-old people in Japan is from 6 to 9. Therefore, the cutoff points at <1, <10 and <20 teeth were used for the grouping.

Clinical information regarding underlying diseases was obtained from a physician's diagnosis or discharge summary. The main underlying diseases in the subjects requiring care were: cerebrovascular disease (n = 165, 50.2%), hypertension (n = 132, 40.1%), heart disease (n = 64, 19.5%), diabetes (n = 30, 9.1%), hepatic disease (n = 4, 1.2%), kidney disease (n = 5, 1.5%), Parkinson's disease (n = 12, 3.6%), orthopedic diseases (n = 44, 13.3%), rheumatism (n = 9, 2.7%) and malignant tumors (n = 10, 3.0%). These diseases were considered to be reliable indicators of systemic problems in the elder-

Table 1 Subject details

Groups of elderly subjects	n	Age years	Male		Female	
			n	%	n	%
Not requiring care	464	72.0±0.3	247	53	217	47
Requiring care	329	83.9±7.5	61	21	230	79
Bedridden status						
Not	31	81.8±6.6	4	13	27	87
Slightly	98	84.0±6.9	21	22	77	78
Moderately	106	83.3±8.7	20	19	86	81
Completely	76	85.2±8.0	18	24	58	76

Age is given as a mean ± SD. All other data are the number of subjects and percent. Bedridden status is described in detail in Subjects and Methods.

ly patients. Informed consent was obtained from all subjects prior to the study and ethical clearance was obtained from the Ethics Committee of the Faculty of Dentistry, Niigata University.

Collection of Dental Plaque Samples

Supragingival plaque samples were collected from the posteroanterior buccal surface of the upper right second premolar and first molar using a cotton swab (Seedswab No. 1, Eiken Chemical Co. Ltd, Tokyo) and then transferred to 1 ml of reduced transport fluid (0.4% agar, 0.15% thioglycollate/phosphate buffered saline) in sterile bottles on ice. For edentulous subjects who used complete dentures, samples were collected from the same regions of the upper right second premolar and first molar of the complete denture. For edentulous subjects not using complete dentures, samples were collected from the residual ridge. Subjects not having any of the above mentioned teeth provided samples from the opposite side or other remaining teeth.

Collection of Pharynx Samples

Samples were collected from the pharynx using a cotton swab (Seedswab No. 1), then transferred into 1 ml of reduced transport fluid in sterile bottles on ice.

Identification of Bacteria and Fungi

The plaque and pharynx samples were taken in transport fluid to the Biomedical Laboratory (Tokyo, Japan) where the isolation frequencies of bacteria and fungi in each were identified using a culture procedure [18]. The samples were poured directly onto chocolate agar, blood agar, OPA staphylococcus and drigalski agar plates (Nippon Becton Dickinson Co. Ltd, Tokyo, Japan). The plates were incubated in an atmosphere of 5% CO_2 at 37 °C for 24–48 h. Representative microbial colonies from each plate were gram stained and isolated by identification using their characteristic appearance, hemolytic, catalytic reaction and oxidase reactions [19]. Bacteria and fungi known to be responsible for pneumonia were chosen for study and are listed in table 2. These colonies were suspended in 1 ml of 0.5% saline, then gently shaken and tested in microbial detection kits [18].

Statistical Procedures

Data for those subjects requiring and not requiring care were compared by a χ^2 test for equal and unequal variations. p values of <0.05 were considered significant.

Table 2 Bacteria and fungi detected in plaque and pharynx samples

Coagulase negative staphylococcus
Staphylococcus aureus
(MSSA, MRSA)
Streptococcus pneumoniae
Streptococcus anginosus
β Hemolytic streptococcus (type A)
β Hemolytic streptococcus (type B)
β Hemolytic streptococcus (type C)
β Hemolytic streptococcus (type D)
Enterococcus faecalis
Enterococcus faecium
Citrobacter freundii
Comamonas acidovorans
Enterobacter spp.
Enterobacter aerogenes
Enterobacter cloacae
Flavobacterium meningosepticum
Acinetobacter calcoaceticus
Haemophilus influenzae
Haemophilus parainfluenzae
Klebsiella spp.
Klebsiella oxytoca
Klebsiella pneumoniae
Klebsiella ozaenae
Moraxella catarrhalis
Morganella morganii
Proteus mirabilis
Pseudomonas spp.
Pseudomonas aeruginosa
Pseudomonas cepacia
Serratia marcescens
Xanthomonas maltophilia
Bacillus cereus
Candida albicans
Candida glabrata
Candida tropicalis
Candida parapsilosis

MSSA = Methicillin susceptible *S. aureus*; MRSA = methicillin resistant *S. aureus*.

Results

Isolation Frequency of Bacteria from Dental Plaque and Pharynx Samples

The isolation frequencies of microbial pathogens from dental plaque and pharynx samples from the patient and control groups are shown in table 3. *Candida albicans*, *Enterobacter cloacae* and *Pseudomonas* spp. from dental plaque samples were significantly higher in subjects requiring care than in control subjects: 133/329 (40%) vs. 135/464 (30%), p < 0.05; 53/329 (16%) vs. 26/464 (6%), p < 0.01; 41/291 (14%) vs. 27/464 (6%), p < 0.01, respectively. In the pharynx samples, *C. albicans* and *Klebsiella pneumoniae* were isolated significantly more often in those requiring care: 83/215 (39%) vs. 101/453 (27%), p < 0.05 and 35/215 (16%) vs. 40/453 (9%), p < 0.01, respectively. In contrast, *Acinetobacter calcoaceticus* from dental plaque samples and *Pseudomonas* spp. from pharynx samples had significantly lower frequencies in the elderly requiring care: 8/329 (2%) vs. 41/464 (0%), p < 0.01 and 7/253 (3%) vs. 35/464 (8%), p < 0.01, respectively. There