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ORIGINAL ARTICLE

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

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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 (type IVa)	1	+	+	+	+
JCSC 4788 (type 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.

addition to the clinical isolates, 21 reference strains listed in Table 1 were used in the current study.

Bacterial colonies on agar plates were suspended in phosphate-buffered saline (PBS) (Invitrogen), and the cell numbers were determined using Petroff-Hauser counting chamber and phase-contrast microscope. For determination of detection limits, ten-fold serial dilutions of cultivated cells (10–10^5 cells per tube) in PBS were prepared and subjected to DNA extraction.

Culture conditions

MRSA and MRSE were cultured on mannitol-salt oxacillin (6 mg ml^{-1}) agar plate (MSO agar: Nissui, Co., Ltd.) at 37°C for 48 h. Methicillin-susceptible *S. aureus* (MSSA) and methicillin-susceptible *S. epidermis* MSSE were grown on the mannitol-salt agar (Nissui, Co., Ltd.). *Fusobacterium nucleatum* and *Porphyromonas gingivalis* were cultured in modified general anaerobic medium (GAM) broth (Nissui Seiyaku Inc.), and *A. actinomycetemcomitans* was cultivated in brain heart infusion broth (Becton, Dickinson and Company) supplemented with 0·5% yeast extract (Becton, Dickinson and Company) and 0·4% sodium bicarbonate. *Streptococcus salivalius*, *S. sanguinis*, *S. mutans* and *E. faecalis* were cultivated in brain heart infusion broth supplemented with 0·5% yeast extract. *E. coli* was grown in Luria–Bertani (LB) broth.

Clinical samples

Eighty-seven dependent elderly hospitalized patients [mean age (year): 70·84 ± 11·52] in Mannari Hospital (Okayama city, Japan) participated in this study. The patients did not have a medical history of MRSA-infection and were routinely received professional oral care. During the care, 28 dental plaque samples and 59 sputum samples were collected from the surface of tooth and oro-pharyngeal swabs, respectively. DNA was extracted promptly from the clinical samples and was subjected to *mecA* and *spa* detection. The sampling and clinical studies were approved by Okayama University Hospital Ethics Committee (approved NO. 439).

DNA extraction

InstaGene Matrix (Bio-Rad) was used for DNA extraction from cultivated strains, clinical dental plaque and sputum samples according to manufacturer's instructions. Briefly, bacterial samples were suspended in PBS, and were pelleted by centrifugation at 10 000 *g* for 15 min and resuspended in 100 μl of InstaGene Matrix. The suspension was incubated at 56°C for 30 min and then 100°C for 8 min. After the incubation, the suspension was centrifuged and 2 μl of the resulting supernatant was used as template for the LAMP and the conventional PCR.

Detection of *mecA* and *spa* by PCR

PCR amplification of the *mecA* and *spa* gene was accomplished with the specific primers under the conditions as described previously (Hiramatsu *et al.* 1992; Shopsin *et al.* 1999). The sequences of those primers were as follows, *mecA* forward (mA1): 5′-TGCTATCCACCCTCAAACA GG-3′, *mecA* reverse (mA2): 5′-AACGTTGTAACCACCC CAAGA-3′, *spa* forward (1095F): 5′-GACGATCCTTCAG TGAGCAAAG-3′, *spa* reverse (1517R): 5′-GCAGCAATTT TGTCAGCAGTA-3′. The PCR products were electrophoretically detected on 2% agarose gels.

LAMP primers

The candidates of LAMP primer sets were nominated from the nucleotide sequence of *mecA* and *spa* by Primer Explorer version 2 (Fujitsu) at the Net Laboratory website (http://www.venus.netlaboratory.com/partner/lamp/index.html). Nucleotide sequences of *mecA* (AB033763, D86934, AB037671, AB063172, AB063173, AB096217) and *spaA* (X61307, U54636, M18264, J01786) were obtained from the GenBank at the National Center for Biotechnology Information (NCBI) website and were aligned by GENETYX ver. 8 (Genetyx, Tokyo, Japan). A set of four primers, a forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3) were selected for each target gene from the candidate primer sets, possibly not to include the polymorphic regions (Table 2).

LAMP reaction

The LAMP reaction was carried out using a Loopamp DNA amplification kit (Eiken Chemical) in a 25-μl volume. The reaction mixture contained 40 pmol each of

Table 2 Primers of LAMP for *mecA* and *spa*

Target gene	Primer	Sequence
mecA	F3	5′-aagatggcaaagatattcaact-3′
	B3	5′-aggttcttttttatcttcggtta-3′
	FIP	5′-acctgtttgagggtggatagcatgatgctaaagttcaaaagagt-3′
	BIP	5′-gcacttgtaagcacaccttcacttcgttactcatgccatac-3′
spa	F3	5′-ggtgatacagtaaatgacattgc-3′
	B3	5′-acgctaatgataatccaccaa-3′
	FIP	5′-cttgaccaggtttgatcatgttttttactgctgacaaaattgctg-3′
	BIP	5′-aaccatgcagatgctaacaaagctacagttgtaccgatgaatgg-3′

FIP, forward inner primer; BIP, backward inner primer; LAMP, loop-mediated isothermal amplification.

FIP and BIP, 5 pmol each of F3 and B3 primers, 2 μl of template, 1 μl of *Bst* DNA polymerase (8 units) and 12·5 μl of reaction mix prepared in the kit. The reaction was carried out under isothermal condition at 64°C for 60 min. After the incubation, the reaction was terminated by heating the reaction mixture at 80°C for 5 min.

Detection of LAMP products

The LAMP product was detected by naked-eye inspection or agarose gel electrophoresis. For naked-eye detection, 1·0 μl of 10^{-1}-diluted SYBR Green I (Takara Bio, Otsu, Japan) was added to the reaction mixture, and the colour change was observed under natural light. For the electrophoretic analysis, 2 μl of reaction mixture was loaded on 2% agarose gel. The gels were stained with ethidium bromide (50 μg ml^{-1}) and assessed photographically under UV light (302 nm).

Results

Specificity of LAMP

Specificity of the LAMP for *mecA* and *spa* was examined using 29 cultivated strains (Table 1). DNA samples were extracted from 10^5 cells of each strain, and the LAMP products were electrophoretically detected. The representative results of electrophoretic detections were shown in Fig. 1. The LAMP for *mecA* successfully amplified the DNA from the strains of MRSA and MRSE. No amplicons were seen in other tested strains. Successful LAMP reactions were also seen with the primer set for *spa* and template DNA from MRSA and MSSA strains. The *mecA* and *spa* were simultaneously detected by conventional PCR using the same DNA templates. The results of PCR were consistent with the results of LAMP (Fig. 1 and Table 1).

Detection limit of LAMP and conventional PCR

For the determination of detection limit, the LAMP reactions were performed using serial diluted DNA templates of MRSA strain (NCTC 10442), and the amplicons were detected by both agarose gel electrophoresis and naked-eye inspection. The results of electrophoretic detection were shown in Fig. 2. The detection limit of the LAMP for *mecA* was $<10^2$ cells (Fig. 2a), while the detection limit for *spa* was <10 cells (Fig. 2b).

The results of naked-eye inspection were shown in Fig. 3. The colour of reaction mixture of LAMP for *mecA*

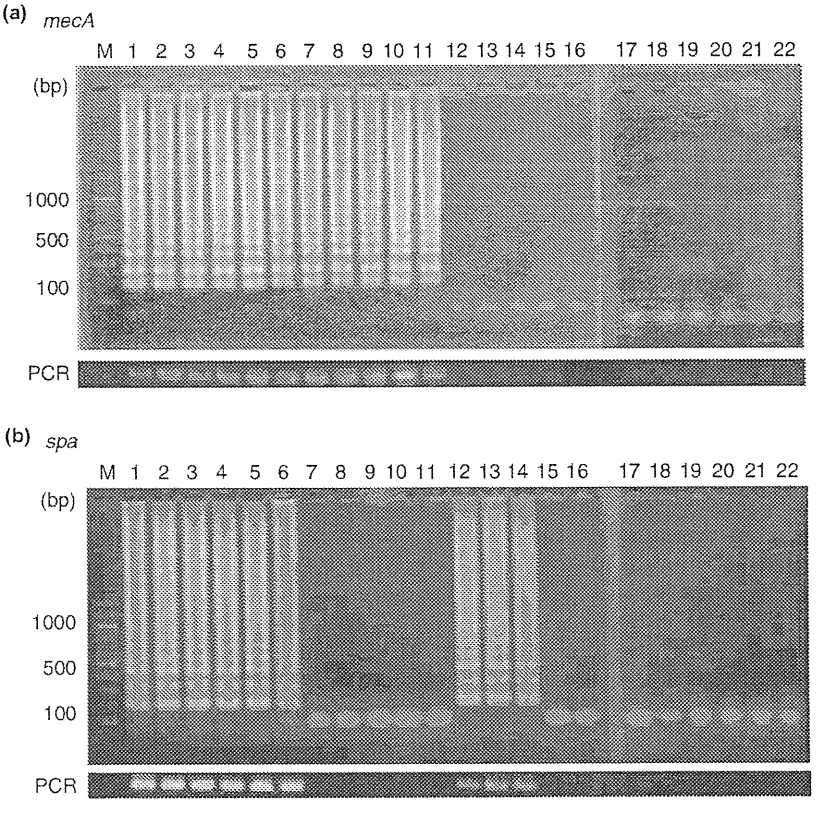

Figure 1 Specificity tests of the LAMP for *mecA* (a) and *spa* (b). All bacterial species prepared in the current study were tested, and the representative results were shown. The *mecA* and *spa* were simultaneously detected by PCR, and the amplicons were shown below the LAMP results. Lane M: DNA size marker, Lane 1–6: Methicillin-resistant *Staphylococcus aureus* (MRSA) (NCTC 10442, N 315, 85/2082, JCSC 4744, JCSC 4788 and WIS), Lane 7–11: methicillin-resistant *Staphylococcus epidermidis* (MRSE) (clinical isolates), Lane 12–14: MSSA (NBRC 14462, NBRC 15 035 and FDA 209), Lane 15–17: MSSE (ATCC 155, ATCC 12228 and ATCC 14990), Lane 18: *S. mutans* ATCC 700610, Lane 19: *P. gingivalis* FDC 381, Lane 20: *E. coli* ATCC 25922, Lane 21: *A. actinomycetemcomitans* ATCC 29523, Lane 22: water (negative control).

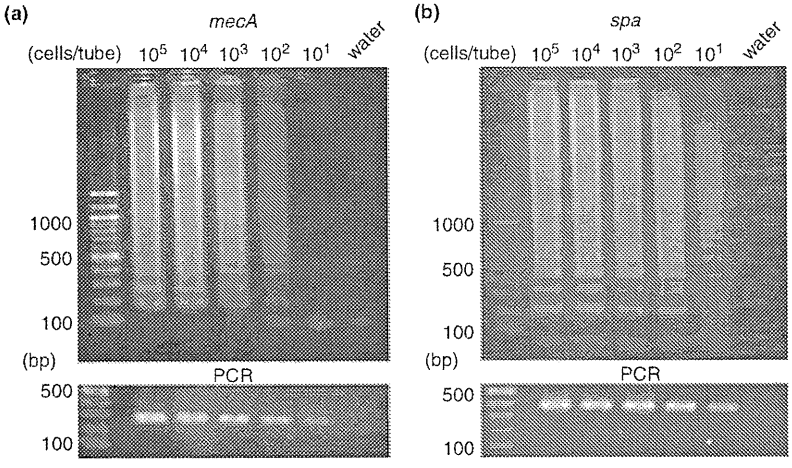

Figure 2 Detection limits of the loop-mediated isothermal amplification (LAMP) for *mecA* (a) and *spa* (b). DNAs were extracted from serial diluted cells (10–10^5 cells) of Methicillin-resistant *Staphylococcus aureus* (MRSA) (NCTC 10442) and were used as templates. The detection limits of the conventional PCR methods were simultaneously examined and were shown below.

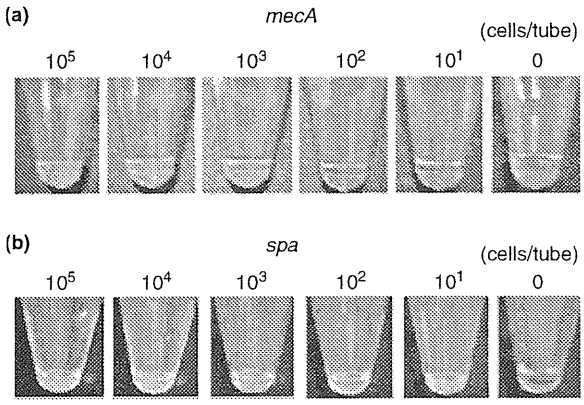

Figure 3 Naked-eye inspection of the LAMP for *mecA* (a) and *spa* (b). The original orange colour of SYBR green I turned to be green in the positive reaction mixture.

exhibited green by the addition of SYBR Green I when the mixture contained DNA templates from more than 10^3 cells, whereas original orange colour did not change when the cell number was less than 10^2 (Fig. 3a). Similarly, naked-eye inspection was feasible for detection of *spa* with the detection limit of ten cells (Fig. 3b). The detection limits of the conventional PCR method were less than ten cells for both *mecA* and *spa* (Fig. 2).

Application of LAMP to clinical samples

The LAMP for *mecA* and *spa* were applied to the clinical plaque and sputum samples. The conventional PCR methods were simultaneously performed, and the results of both methods were compared (Table 3 and 4). By the LAMP methods, *mecA* was detected in 6 plaque samples (21·4%), and *spa* was detected in 7 (25·0%) of the 28 plaque samples. The LAMP method demonstrated 100% agreement with the conventional PCR in analysing the plaque samples (Table 3).

In analysing the sputum samples, the LAMP detected *mecA* in nine samples (15·3%), whereas PCR detected the gene in 13 samples (22·0%). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the LAMP for *mecA* were 69·2, 100, 100, 92·0%, respectively. The LAMP for *mecA* showed 93·2%

Table 3 Detection of *mecA* and *spa* from clinical plaque samples

	PCR assay						
LAMP assay	Positive	Negative	Percent agreement (kappa value)	Sensitivity	Specificity	PPV*	NPV†
mecA							
Positive	6	0	100% (1·00)	100%	100%	100%	100%
Negative	0	22					
spa							
Positive	7	0	100% (1·00)	100%	100%	100%	100%
Negative	0	21					

LAMP, loop-mediated isothermal amplification.
*Positive predictive value.
†Negative predictive value

Table 4 Detection of *mecA* and *spa* from clinical sputum samples

LAMP assay	PCR assay		Percent agreement (kappa value)	Sensitivity	Specificity	PPV*	NPV†
	Positive	Negative					
mecA							
Positive	9	0	93·2% (0·78)	69·2%	100%	100%	92·0%
Negative	4	46					
spa							
Positive	9	0	86·4% (0·62)	52·9%	100%	100%	84·0%
Negative	8	42					

LAMP, loop-mediated isothermal amplificationl.
*Positive predictive value.
†Negative predictive value.

(κ = 0·78) agreement with the conventional PCR method. The *spa* was detected by the LAMP in 9 (15·3%) of the 59 sputum samples, whereas 17 (28·8%) samples were positive in PCR detection (Table 4). Sensitivity, specificity, PPV and NPV of the *spa* LAMP were 52·9, 100, 100, 84·0%, respectively, and the per cent observed agreement with the conventional PCR was 86·4% (κ = 0·62).

Discussion

As opportunities of oral care for elderly or hospitalized patients are increasing, dentists must give care to the distribution of antibiotic resistance such as MRSA. PCR is a rapid molecular technique for the microbiological diagnosis. However, the method is sometimes difficult to perform because of the lack of facilities, especially in case of home-nursing patients. Therefore, it is important for dentists to have a rapid and simple detection method for antibiotic resistance. In the current study, LAMP method was applied to the detection of *mecA* and *spa*.

The LAMP reactions were successfully accomplished within 60 min. Culture methods require 24–48 h until a result is known, whereas 2–4 h of reaction time is required for the PCR assay. When compared to these methods, the LAMP for *mecA* and *spa* demonstrated a great advantage in the rapidity. However, among PCR-based methods, real-time PCR with LightCycler System had also an advantage in the rapidity (Shrestha *et al.* 2002), as the capillary is designed to have an optimal surface-to-volume ratio to ensure rapid temperature control. By using SYBR Green chemistry, the reaction can be performed at similar cost with LAMP method ($4–5 per reaction), although an expensive equipment is required. Clinicians need to select appropriate methods according to the purpose of the examination and circumstance of the clinics.

The LAMP is a highly specific method attributable to a set of two specially designed inner and outer primers that recognize six distinct sequences (Notomi *et al.* 2000). As expected, specificity test using the cultured cells demonstrated the high specificity of the LAMP for both of *mecA* and *spa*. In addition, in analysing plaque samples, the LAMP demonstrated complete concordance with the conventional PCR method. As hundreds of bacterial species exist in oral cavity and dental plaque (Paster *et al.* 2001), these results may also support the specificity of the LAMP established in the current study.

The detection limits of the LAMP for *mecA* and *spa* by agarose gel electrophoresis were $<10^2$ cells and 10 cells, respectively, while the detection limits of the PCR methods were less than ten cells for both targets. The LAMP methods generally demonstrate equivalent or higher sensitivity with the PCR methods (Maeda *et al.* 2005; Miyagawa *et al.* 2008). However, the detection limit for *mecA* was inferior to that of the conventional PCR method because of unknown reason. The LAMP reaction can be accelerated, and higher sensitivity would be expected by using additional primer, termed loop primer (Nagamine *et al.* 2002). Redesign of the LAMP primer set including the loop primer may improve the detection limit for *mecA*.

The naked-eye inspection of LAMP for *spa* demonstrated the equal sensitivity to the electrophoretical detection (10 cells per tube), while the sensitivity of the LAMP for *mecA* declined to 10^3 cells. By increasing the reaction time to 90 min, the naked-eye inspection for *mecA* improved the sensitivity to 10^2 cells (data not shown). The quite simple and rapid eye inspection may be useful for application of the LAMP methods.

In analysis of the plaque samples, the LAMP and PCR demonstrated identical results, suggesting the clinical applicability of the method for plaque samples. However, in analysis of the sputum samples, the sensitivity of the LAMP was considerably declined in both *mecA* and *spa* detection. DNA extraction performed in the current study was based on a simple boiling method. Comparing to the plaque samples, the sputum samples may contain larger amount of inhibitors for the LAMP reaction. The DNA extraction procedure should be reconsidered. The use of lysostaphin (Schindler and Schuhardt 1964) will be a

good strategy for the DNA extraction. The analyses of clinical samples revealed the carrier of antibiotic resistance and verify the significance of examinations in clinical fields of dentistry.

Each LAMP methods for *mecA* and *spa* were accomplished in an hour with high specificity and sensitivity. In addition, through the naked-eye inspection, the LAMP obtained great advantages in simplicity and rapidity. Although further experiments will be required for the examination of sputum samples, the LAMP methods established in the current study have a potential to be beneficial tools for the detection of *mecA* and *spa*.

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Original

Attitudes Towards HIV-Infected Patients, Knowledge Related to HIV/Universal Precautions, and Infection Control Practices of Japanese Dentists

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Abstract: The aim of this study was to explore factors influencing attitudes towards HIV-infected patients, knowledge related to human immunodeficiency virus (HIV)/universal precautions, and infection control practices (ICPs) of Japanese dentists, and to analyze the relationship between the attitude toward HIV-infected patients and knowledge related to HIV/universal precautions, and infection control practices in a population of Japanese dentists. Data on attitudes towards HIV-infected patients, knowledge related to HIV/universal precautions, and ICPs were obtained from 3,316 dentists in Aichi Prefecture, Japan, through questionnaires, which were then statistically analyzed. Overall, an age of 49 years or younger, specialist in oral surgery, and seeing 36 or more patients per day were positive significant predictors for attitudes towards HIV-infected patients, knowledge related to HIV/universal precautions, and ICPs. Those aged 49 years or younger were more likely to report desirable responses for all attitude items than older dentists. Those seeing 36 or more patients per day were more likely to report desirable ICPs in all but one item than those seeing 35 or less. In addition to being younger, specialty in oral surgery, seeing 36 or more patients per day, and having a higher knowledge index were significant positive associating factors for a higher infection control practice index. Dentists who are younger, have a specialty in oral surgery, or see more patients per day showed positive attitudes for dental care provision toward HIV-infected patients, greater knowledge related to HIV/universal precautions, and better compliance for ICPs. Knowledge related to HIV/universal precautions was positively related to ICPs.

Key words: **Dentist, Infection control practice, Attitude, Knowledge, Infectious disease**

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Introduction

The number of newly reported human immunodeficiency virus (HIV)-infected patients and newly reported AIDS patients has been increasing every year (HIV infection: 397 in 1997 to 1,021 in 2009; AIDS: 250 in 1997 to 431 in 2009), and, at present, there are more than 10,000 HIV-infected patients in Japan[1]. Furthermore, the number of hepatitis C virus (HCV) carriers is estimated to comprise about 2% of the entire population in Japan, the highest incidence among developed countries. Given these circumstances, dental care for patients with infectious diseases is of marked concern in Japan. Japanese dentists now have a higher risk of exposure to pathogens via blood or saliva while providing dental care than ever before. The provision of dental health care to persons infected with HIV has been deemed an ethical responsibility[2-5]. The necessity for dentists to be willing to provide dental care for patients with infectious disease and to perform infection control practices (ICPs) has been increasing.

In many countries, there have been reports documenting the status of dentists' attitudes, knowledge of HIV/AIDS patients, and ICPs[6-19]. Factors influencing attitudes and ICP compliance have been analyzed[20-27]. However, little information is available about the influence of demographic factors on dentists' attitude toward HIV-infected patients, knowledge related to HIV/

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universal precautions, and ICPs, or on the association among attitudes, knowledge about infectious diseases, and ICPs in Japan's dentists. To our knowledge, only one study has investigated the association between age and ICPs[28]. In recent years, a general clinical training system for dental school graduates has been introduced in Japan, and the Japan Dental Association has promoted training for practitioners performed by each local Dental Association. The necessity for introducing ICPs in these training programs is now proposed.

The present study has explored the factors influencing attitudes towards HIV-infected patients, knowledge related to HIV/universal precautions, and ICPs in a population of Japanese dentists. Furthermore, we analyzed the relationship between attitude, knowledge, and ICPs.

Study Population and Method

1. Study population and procedure for data collection

This study was conducted involving dentists from Aichi Prefecture between August and October 2008. Since the cumulative HIV infection rate in Aichi Prefecture was 6.05/100,000 of the population, which is similar to that of Japan (6.47/100,000 of the population), we considered Aichi Prefecture as representative of Japan in terms of HIV infection. A self-administered questionnaire was sent to all 3,316 directors of dental offices listed as members of the Aichi Prefecture Dental Association. The self-administered questionnaire was accompanied by a letter of endorsement signed by the President of the Aichi Prefecture Dental Association and a letter of introduction signed by the research team emphasizing the importance of this study and ensuring the anonymity of responders. One thousand nine hundred and twenty-five (58.1%) were returned.

2. Sociodemographic factors

Sociodemographic factors included gender, age, specialty in oral surgery, and the number of patients treated per day. The distribution of subjects by sociodemographic factors is shown in Table 1. Under Japan's dental health service system, dentists are able to establish private dental offices offering services in the specialties of dentistry, oral surgery, pedodontics, or orthodontics. In terms of specialty in oral surgery, we classified dentists into two groups, 'specialists' and 'general dentists'. 'Specialists' were dentists who have established private dental offices which include oral surgery as a specialty and offer relatively difficult oral surgery treatment as well as restorative and prosthodontic treatments. 'General' dentists were those who have established private dental offices that do not include oral surgery as a specialty and mainly perform restorative and prosthodontic treatment, as well as basic oral surgery such as tooth extraction. The number of patients treated per day was counted for each private dental office.

Table 1 The distribution of subjects by sociodemographic factors

Variable	Number (percentage)
Gender	
Male	1,809 (95.1)
Female	94 (4.9)
Age	
-39	176 (9.4)
40-49	516 (27.5)
50-59	776 (41.3)
60-	410 (21.8)
Specialty of oral surgery	
Specialist	206 (11.0)
General	1,665 (89.0)
Number of patients a day	
≦15	292 (16.9)
16-25	548 (31.6)
26-35	467 (27.0)
36-45	251 (14.5)
46<	174 (10.0)

Missing data were excluded.

3. Variables measured

Dentists were asked to complete the questionnaire. Items of the questionnaire covered attitude (4 items), knowledge (3 items), and ICPs (9 items). Each item was dichotomously categorized as follows: attitude items (agree, disagree), knowledge items (yes, no), and ICP items (yes, no).

Summated rating scales were constructed for indices of attitude, knowledge, and ICPs. Positive behaviors were assigned a score of 1 and negative behaviors were assigned a score of 0. Correct knowledge was assigned a score of 1, and lack of knowledge was scored 0. If infection control practices were employed, the score

Table 2 The score of responses to questions concerning attitudes, knowledge, and infection control practice

Variable	Category	Number (percentage)
Attitude		
Willing to treat HIV patients in my practice	Agree	414 (22.1)
Willing to treat HIV patients in another practice	Agree	577 (31.8)
Refusal of HIV patients is ethically wrong	Agree	1,173 (63.3)
If I treat patients with HIV or AIDS, my other patients may be reluctant to continue in my care	Agree	1,258 (67.8)
Knowledge		
I know the meaning of Universal Precautions	Yes	258 (15.9)
Do you think HIV is transmitted via saliva contaminated with blood	Yes	1,569 (84.4)
I know the meaning of HAART	Yes	75 (4.0)
Infection control practice		
When treating patients, do you:		
Always wear protective eyewear	Yes	636 (33.6)
Always wear mask for treatment	Yes	1,689 (97.3)
Always wear gloves for treatment	Yes	1,248 (72.0)
Exchange handpiece for each patient	Yes	375 (21.7)
Provide education for preventing infection of clinical staff	Yes	1,448 (83.8)
Prepare office infection control manual	Yes	959 (55.9)
Usually participate in clinical lectures for infection control	Yes	1,353 (79.5)
HBV immunization (dentist)	Yes	1,130 (65.5)
HBV immunization (clinical staff)	Yes	343 (20.0)

Not all respondents completed every item.

was 1, and if they were not used, the score was 0. Thus, the sum score could range from 4 to 0 for attitude, 3 to 0 for knowledge, and from 9 to 0 for ICP. Each index was categorized into 'high' and 'low' as follows:

Attitude index 'high': 3 or more
'low': 2 or less
Knowledge index 'high': 2 or more
'low': 1 or less
ICP index 'high': 5 or more
'low': 4 or less

4. Ethics

Ethical approval for the study was provided by the Aichi Prefecture Dental Association. A consent form was used to obtain informed consent and clarify that no direct benefit could be expected from participating in the study. All data collected were confidential and anonymous.

5. Data analysis

To analyze the associations of sociodemographic factors with attitudes, knowledge, and ICPs, logistic regression models were used. The dependent variables consisted of each item of attitude, knowledge, and ICPs. Sociodemographic factors were used as independent variables for the analysis. Furthermore, we analyzed models by introducing the Attitude index (high/low) and Knowledge index (high/low), in addition to sociodemographic factors with dependent variables, the ICP index (high/low). Statistical analyses were carried out using SPSS. ver. 12. Differences at the 0.05 level were considered significant.

Results

1. Attitudes, knowledge, and ICPs

Table 2 shows the distribution of all subjects by each item of attitude, knowledge, and ICPs. With regard to attitude, only 22.1 and 31.8% of respondents reported a willingness to treat HIV-infected patients in their practice and in another clinics, respectively, although 63.3% reported they should not refuse to treat HIV-infected patients. With regard to knowledge, only 15.9 and 4.0% knew the meaning of universal precautions and highly active antiretroviral therapy (HAART), respectively,

Table 3 Multiple regression of gender, age, specialty, and number of patients per day with attitude and knowledge toward HIV-infected patients and infection control practice

	Gender	Age	Specialty	Number of patients a day
Attitude				
Willing to treat HIV patients in my practice		2.7 (2.1-3.4) <0.001	1.7 (1.3-2.5) 0.001	
Willing to treat HIV patients in another practice		2.2 (1.7-2.7) <0.001	1.9 (1.4-2.7) <0.001	1.4 (1.1-1.7) 0.009
Refusal of HIV patients is ethically wrong		1.4 (1.1-1.7) 0.005		1.3 (1.1-1.6) 0.014
If I treat patients with HIV or AIDS, my other patients may be reluctant to continue in my care	0.6 (0.3-0.8) 0.034	0.6 (0.5-0.8) <0.001		
Knowledge				
I know the meaning of Universal Precautions		1.8 (1.3-2.4) <0.001	2.2 (1.5-3.2) <0.001	1.5 (1.1-2.0) 0.011
I think HIV is transmitted via saliva contaminated with blood				
I know the meaning of HAART		2.2 (1.3-3.7) 0.003	3.8 (2.2-6.7) <0.001	
Infection control practice				
Always wear protective eyewear for treatment		1.3 (1.0-1.6) 0.034	1.5 (1.1-2.0) 0.021	1.4 (1.2-1.8) 0.001
Always wear mask for treatment	4.0 (1.6-10.1) 0.003	3.0 (1.3-7.0) 0.010		
Always wear gloves for treatment	0.5 (0.2-0.9) 0.011	3.3 (2.5-4.3) <0.001		2.1 (1.7-2.7) <0.001
Exchange handpiece for each patient		1.6 (1.2-2.0) <0.001	1.5 (1.0-2.1) 0.026	1.7 (1.3-2.2) <0.001
Education for preventing infection of clinical staff	0.3 (0.1-0.7) 0.010		2.0 (1.1-3.4) 0.017	2.4 (1.8-3.2) <0.001
Preparing office infection control manual				1.8 (1.4-2.2) <0.001
Participation in clinical lecture for infection control		0.7 (0.5-0.9) 0.001		1.5 (1.4-2.1) 0.001
HBV immunization (dentist)		2.7 (2.1-3.4) <0.001	1.5 (1.4-2.1) 0.019	1.6 (1.3-1.9) <0.001
HBV immunization (clinical staff)			1.5 (1.0-2.1) 0.035	1.6 (1.3-2.1) 0.010

Not all respondents completed every item.
Upper: Odds ratios (95% confident intervals), bottom: p-value

compared to 84.4% who knew that HIV is transmitted via saliva contaminated with blood. Regarding ICPs, 97.3% reported wearing masks during dental treatment and 83.8% responded that they provide education about infection prevention for clinical staff. The least commonly reported ICP was HBV immunization for dental staff (20.0%) compared to HBV immunization for dentists (65.5 %).

2. Association of sociodemographic factors with attitude, knowledge, and ICPs using logistic regression analysis

Table 3 shows the associations of sociodemographic factors with attitude, knowledge, and ICPs using logistic regression analysis. Each item of attitude, knowledge, and ICPs was introduced into the regression models.

1) Attitude

Being aged 49 or younger and specialty in oral surgery were significant predictors of being willing to treat HIV-infected patients in their practice (age: OR=2.7, specialty: OR=1.7) and other clinics (age: OR=2.2, specialty: OR=1.9). The number of patients seen per day was also a significant predictor of being willing to treat HIV-infected patients in other clinics (OR=1.4). Age and the number of patients seen per day were significantly associated with the belief that refusing dental treatment for HIV patients is ethically wrong (age: OR=1.4, number of patients: OR=1.3). Men and the younger age group were less likely to fear that other patients may be reluctant to continue in their care when they treat HIV-infected patients (OR=0.6).

2) Knowledge

Age and specialty in oral surgery were significant predictors of being familiar with universal precautions (age: OR=1.8, specialty: OR=2.2) and HAART (age: OR=2.2, specialty: OR=3.8). The number of patients seen per day was significantly associated with being familiar with universal precautions (OR=1.5).

3) Infection control practice

All explanatory variables except gender were associated with wearing protective eyewear, changing handpieces after each treatment, and receiving HBV immunization (dentists). Being female, being younger, and seeing a greater number of patients were associated with being more likely to wear gloves (gender: OR=0.5, age: OR=3.3, number of patients: OR=2.1). Education for clinical staff on preventing infection was associated with all explanatory variables except age. Clinical staff vaccinated against HBV showed an association with specialty in oral surgery (OR=1.5) and number of patients a day (OR=1.6). Being male and younger were associated with an increased likelihood of wearing a mask (gender: OR=4.0, age: OR=3.0). Being older and seeing a greater number of patients were associated with being more likely to receive educational lectures (age: OR=0.7, number of patients: OR=1.5). Preparation of an office infection control manual was associated only with the number of patients treated a day (OR=1.8).

Table 4 Multiple regression of gender, age, specialty, number of patients per day, attitude index, and knowledge index with infection control practice index

Variables	OR	95%CI	p-value
Gender	1.1	0.6-2.0	0.806
Age	1.5	1.2-2.0	0.002
Specialty	1.5	1.0-2.1	0.041
Number of patients	1.6	1.2-2.1	<0.001
Attitude	1.3	0.9-1.7	0.135
Knowledge	2.2	1.6-3.1	<0.001

Not all respondents completed every item.

3. Association of infection control practice with attitude and knowledge

We explored factors associated with a high ICP index using logistic regression analysis with explanatory factors consisting of the Attitude index, Knowledge index, and four sociodemographic variables (gender, age, specialty, and number of patients) (Table 4). Having a high Knowledge index, being younger, specialty in oral surgery, and seeing more patients were significantly positively associated with a high ICP index score.

Discussion

Relationships between sociodemographic factors and attitude, knowledge, and ICPs were analyzed using multiple logistic regression analyses. Female dentists were more likely to think that patients would be reluctant to continue in their care if they treated HIV-infected patients. They were also more likely to wear gloves and provide their clinical staff with education about preventing infection than men, and were less likely than men to wear masks. In McCarthy's report[23], women were more likely to wear gloves, which is consistent with our findings; however, in contrast with our results, they found that women were more likely to wear masks and less likely to fear that other patients may be reluctant to continue in their care if they treated HIV-patients.

Younger dentists had a significantly or relatively higher proportion of desirable responses than older dentists in most items on attitudes and ICPs. Increased numbers of HIV-infected patients in Japan and the advent of new infectious diseases have led to a stronger emphasis on the prevention of infection in undergraduate education. Younger dentists who have been

better educated about the prevention of infection during their studies are considered to have more knowledge about ICPs and better attitudes towards the prevention of infectious diseases than older dentists. Similar results have been reported regarding the routine use of gloves and HBV immunization of dentists[22,27]. Several studies have reported that younger dentists were more willing to provide dental care for patients with HIV infection[22,27,29]. Our results are consistent with these studies.

Overall, specialists in oral surgery exhibited a higher proportion of desirable attitudes, correct knowledge, and compliance with desirable ICPs than general dentists, confirming the findings of another report[27]. Most specialists in oral surgery in Japan have undergone post-graduate training at a university or hospital covering the prevention of infection during surgical treatment and treatment of patients with infectious diseases. On the other hand, dentists who did not major in oral surgery had fewer opportunities to learn about infectious diseases and ICPs. Lueveswanij *et al.* reported that educational intervention could change knowledge, attitudes, and ICPs of oral health personnel with regard to AIDS[30]. These findings suggest that specific education and training about infection control could change attitudes and infection control practices of dental health workers.

Dentists treating a smaller number of patients a day showed less compliance in wearing gloves during treatment, providing education and HBV immunization for clinical staff, and preparing a manual than their counterparts. Some studies reported that dentists who see more patients per day showed favorable compliance with ICPs[7,17]. Our results were consistent with these reports. Dentists treating a smaller number of patients per day may see using gloves during treatment as a financial burden, since a small patient base is linked with a small income. Other studies have reported that some dentists see ICPs as a financial burden[22,23]. However, providing education for staff and preparing infection control manuals are not expensive undertakings. This group showed less willingness to treat HIV-infected patients, suggesting that they feel they cannot afford to invest their time in infectious disease prevention. On the other hand, dentists with many patients may treat a large number with blood-borne pathogens, so they are more likely to be prepared by adopting infection control measures in their practice. These results emphasize the importance of educating dentists who see a small number of patients about employing measures to prevent the spread of infectious diseases.

It has been argued that knowledge and attitudes may influence adults' health behavior more or less over a long period of time[31-34]. Since we hypothesized that favorable attitudes and knowledge would lead to favorable ICPs, we analyzed the association of attitudes and knowledge with ICPs. The knowledge index had a significant association with the ICP index. Among the knowledge items, knowledge for 'universal precautions' and 'HAART' showed a significant relationship with the ICP index (universal precautions: $p<0.001$, HAART: $p<0.001$), which contributed to the association of the Knowledge index with the ICP index. This is of particular importance, given that an understanding of the concept of universal precautions may provide the motivation for dentists to undertake ICPs in their practice. To our knowledge, this is the first report describing the association of knowledge with ICPs in dentists, and further investigation is needed to analyze this association in more detail. The findings of this study revealed a lower compliance with some ICPs (protective eyewear, gloves, office infection control manuals, changing of handpieces, HBV vaccines) compared with data from other countries[7,9,10,13,15,16,22,27,29]. Lower standards of ICPs suggest that dentists need to be re-educated regarding infectious diseases and their management. In particular, groups showing poorer compliance with ICPs, i.e., older or 'general' dentists, should be a target population for such education. Moreover, it is important to motivate dentists seeing a smaller number of patients to develop more of an interest in infection control practices. The significant correlation between knowledge and ICPs suggested that increasing knowledge about infectious diseases and infection control may be of help to improve ICPs. Several studies have reported that educational intervention improved compliance with infection control practices[21] and attitudes towards HIV/AIDS of dentists[24,35,36], which supports the significance of education. One report suggested that using a questionnaire to identify deficiencies in ICPs was advantageous for

designing an education program[30]. The findings of the present study may be useful for the development of an educational program for dentists and under-graduate dental students.

Dentists with a greater awareness of providing dental health care to patients with HIV infection tended to also carry out ICP measures, but no significant association was found between them. This suggests that having such an awareness does not necessarily compel dentists to undertake ICPs. Some reports have documented that an increase in self-efficacy is necessary for dentists to provide dental treatment for patients with AIDS or HIV infection[12, 37, 38]. In general, different factors have been found to be associated with the health-related behavior of adults. Psychological processes related to the treatment of HIV-infected patients and of infection control practices by dentists are considered to be responsible for complex psychosocial mechanisms, and the elucidation of these mechanisms requires much more investigation. The increasing number of patients with infectious diseases highlights the need for dentists to undergo training concerning ICPs, and effective training requires more information on behavioral science concerning dentists' use of ICPs.

On the other hand, compared with a similar survey done in the same population in 2005 (unpublished data), the present study showed a marked increase in the proportion of dentists reporting the preparation of an office infection control manual (55.7 vs. 28.7, respectivery) and provision of education to clinical staff for preventing infection (83.8 vs 39.6, respectively). 'The Revision of the Medical Care Act in 2007' required the reinforcement of systems preventing nosocomial infections in medical institutions. This covers requirements for the preparation of an office infection control manual and the provision of education for clinical staff to prevent infection, and is considered to have contributed to the improvement of these two ICPs. We suggest that the government's public health policy should include requirements for improving dentists' ICPs.

Several reports have described how dentists' compliance with ICPs in other countries has improved over time[6, 9, 36]. Furthermore, a cohort study of Japanese dentists' attitude toward HIV-infected patients and compliance with infection control should be done. We aim to undertake a similar cohort study monitoring Japanese dentists' attitudes toward HIV-infected patients and compliance with infection control measures in the near future.

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日本人歯科医師のHIV感染症患者に対する態度，HIV/ユニバーサルプレコーションに関する知識，院内感染防止対策実施

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概要：本研究の目的は日本の歯科医師における「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」に関与する因子を探索し，さらに，「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」の相互の関連を解析することである．愛知県に開業している歯科医師3,316人に対し「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」についてのアンケート調査を行い，回答のあった人に対し統計的な解析を行った．全体として，「49歳以下」，「口腔外科専攻」，「1日36人以上の患者を診療する」はHIV感染患者への良好な態度，感染に関する高い知識，院内感染防止実施の有意な予測因子であった．49歳以下の歯科医師は50歳以上の歯科医師よりも「HIV感染患者への態度」におけるすべての項目で望ましい行動をとると回答した．1日36人以上の患者を診療する歯科医師は34人以下の歯科医師に比べ，ほとんどの院内感染防止策実施項目で実施状況が良好であった．「HIV感染患者への態度」，「感染に関する知識」，「院内感染防止策実施」それぞれにつき，各項目の回答結果をもとにインデックス化し「院内感染防止策実施」に有意な関連をもつ因子を調べたところ「感染に関する知識」，年齢，口腔外科専攻，患者数が該当した．「49歳以下」，「口腔外科専攻」，「1日36人以上の患者を診療する」はHIV感染患者への良好な態度，感染に関する知識，院内感染防止実施の予測因子であった．「感染に関する知識」は「院内感染防止策実施」と有意な関連を示した．

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Roles of Salivary Components in *Streptococcus mutans* Colonization in a New Animal Model Using NOD/SCID.*e2f1*$^{-/-}$ Mice

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Abstract

Streptococcus mutans plays an important role in biofilm formation on the tooth surface and is the primary causative agent of dental caries. The binding of *S. mutans* to the salivary pellicle is of considerable etiologic significance and is important in biofilm development. Recently, we produced NOD/SCID.*e2f1*$^{-/-}$ mice that show hyposalivation, lower salivary antibody, and an extended life span compared to the parent strain: NOD.*e2f1*$^{-/-}$. In this study we used NOD/SCID.*e2f1*$^{-/-}$ 4 or 6 mice to determine the roles of several salivary components in *S. mutans* colonization *in vivo*. *S. mutans* colonization in NOD/SCID.*e2f1*$^{-/-}$ mice was significantly increased when mice were pre-treated with human saliva or commercial salivary components. Interestingly, pre-treatment with secretory IgA (sIgA) at physiological concentrations promoted significant colonization of *S. mutans* compared with sIgA at higher concentrations, or with human saliva or other components. Our data suggest the principal effects of specific sIgA on *S. mutans* occur during *S. mutans* colonization, where the appropriate concentration of specific sIgA may serve as an anti-microbial agent, agglutinin, or an adherence receptor to surface antigens. Further, specific sIgA supported biofilm formation when the mice were supplied 1% sucrose water and a non-sucrose diet. The data suggests that there are multiple effects exerted by sIgA in *S. mutans* colonization, with synergistic effects evident under the condition of sIgA and limited nutrients on colonization in NOD/SCID.*e2f1*$^{-/-}$ mice. This is a new animal model that can be used to assess prevention methods for dental biofilm-dependent diseases such as dental caries.

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Introduction

Oral streptococci are present in large numbers in dental plaque, which co-interact with the enamel salivary pellicle to form a biofilm on tooth surfaces [1,2]. Streptococcal cell wall components mediate adherence to various salivary receptors [3–6]. The ability of oral streptococci to bind to the salivary pellicle is of considerable etiologic significance in oral disease [4,7]; and is important for biofilm development [8,9]. The glucans synthesized by streptococcal glucosyltransferases convert sucrose into glucan; and provide binding sites through interaction with bacterial cell-associated glucan-binding proteins that promote the accumulation of microorganisms on the tooth surface, and help establish pathogenic biofilms [10,11]. *Streptococcus mutans* plays an important role in biofilm formation on the tooth surface and is a primary causative agent for dental caries [2]. *S. mutans* produces two extracellular glucosyltransferase (Gtfs) that convert sucrose into insoluble glucans [10], where GTF I and GTF SI (water-insoluble glucan) are encoded by *gtfB* and *gtfC*. Animal experiments [12] suggest that the expression of these two *S. mutans gtf* genes is required for maximal virulence in causing dental caries.

It is difficult to extrapolate *in vitro* experimental results to predict the impact of a specific salivary factor in biofilm development. However, the problem facing *in vivo* oral biofilm research is the lack of a natural, reproducible, longitudinal monitoring system permitting the assessment of oral bacterial infection in the same animal throughout the duration of a study. Studies using *S. mutans* infection in animal oral cavities have been performed by feeding the animals powdered Diet 2000 containing unnatural amounts of sucrose (56%). Even when experiments employed feeding a low sucrose content (1 or 5%), longitudinal (more than 2 weeks) feeding with frequent inoculation was performed [13–17]. When these methods were used, *S. mutans* was found to produce a larger amount of insoluble glucan in the oral cavities of mice fed foods containing excess amounts of sucrose. These experiments although interesting do not represent human diet styles.

The mechanical forces of salivary flow and tongue movement tend to dislodge and expel bacteria from tooth surfaces and the oral cavity [18,19]. This controls microbial colonization in the oral cavity as shown with insulin-dependent diabetes mellitus (IDDM), Sjögren's syndrome (SS), and drymouth where these patients suffer from a rapid overgrowth of biofilm and caries that make them highly susceptible to oral infections [20,21]. E2F-1 is a member of

the transcriptional factor controlling the initiation of DNA synthesis [22–24] and subsequent transition of cells from the G0/G1 to S phase in the cell cycle [25,26]. Several recent studies have demonstrated that a mutation of the *e2f1* gene in mice causes enhanced T-lymphocyte proliferation, leading to testicular atrophy, splenomegaly, salivary gland dysplasia, and other types of systemic and organ-specific autoimmunity [27–30]. C57BL/6.*e2f1*$^{-/-}$ mice show high susceptibility to oral streptococci because they do not produce sufficient volumes of saliva and salivary proteins [31]. Further, the combination of E2F-1 deficiency and the NOD gene background induced a rapid progressive development of IDDM and SS compared to NOD mice. This is caused by enhanced auto-reactive Th1-type T cells. NOD.*e2f1*$^{-/-}$ mice do not survive long; therefore they are not suitable for long-term bacterial infection experiments [32]. A recent study using NOD/SCID background E2F-1 deficient mice (NOD/SCID.*e2f1*$^{-/-}$) (T and B cells do not develop to observe E2F-1 function in the NOD background mice without an auto-reactive response) showed E2F-1 may be associated with the differentiation of exocrine cells in the salivary duct [33].

The NOD/SCID.*e2f1*$^{-/-}$ mouse has a decreased saliva volume, lacks sIgA and IgG in the saliva, and has decreased NK cells. This may be a useful mouse for studying oral bacterial infection, colonization, and biofilm formation. These mice have long survival because they do not develop IDDM and SS. Therefore, they may be useful as a model animal for oral bacterial colonization under humanized conditions. Establishment of a humanized experimental system could lead to better understanding of the pathogenic conditions associated with oral bacterial infections and the development of more effective agents for control of bacterial infection associated with oral diseases.

Materials and Methods

Bacterial strains and culture conditions

Streptococcus mutans UA159 was used for colonization study and ELISA. *Actinomyces naeslundii* X600 was used for ELISA as control oral bacteria. All bacteria were grown in an atmosphere of H_2 and CO_2 (GasPack, Becton/Dickinson, Sparks, MD) in Brain Heart Infusion broth (BHI, Difco Laboratory, Detroit, MI) at 37°C.

Animals

NOD/LtJ mice naturally develop IDDM, SS, and dry mouth; and were the parent strain to develop NOD/SCID.*e2f1*$^{-/-}$ mice. They were used as the control to compare *S. mutans* susceptibility to NOD back ground E2F-1$^{-/-}$ mice (NOD.*e2f1*$^{-/-}$) and NOD/SCID back ground E2F-1 heterogeneous (NOD/SCID.*e2f1*$^{+/-}$) and homogeneous deficient NOD/SCID mice (NOD/SCID.*e2f1*$^{-/-}$) [33]. NOD/SCID mice were the parental lines to produce NOD/SCID.*e2f1*$^{-/-}$ mice [33] and were used as control mice in bacterial inoculation experiments. Heterozygous NOD/SCID.*e2f1*$^{+/-}$ mice were bred to generate NOD/SCID.*e2f1*$^{-/-}$ mice. Three types (+/+, +/− and −/− of *e2f1*) of NOD/SCID mice were screened using PCR [33]. All strains were female, 4 months of age and were maintained in accordance with the guidelines for the Care and Use of Laboratory Animals from the National Institute of Infectious Diseases. Experimental protocols (#209125, 210110, and 21124) were approved by the National Institute of Infectious Diseases Animal Resource Committee.

Human saliva collection

Saliva samples were collected from volunteers with good oral health, after stimulation by chewing paraffin gum. The volunteers refrained from eating, drinking, and brushing for at least 2 h prior to collection. The saliva was placed into ice-chilled sterile bottles for 5 min; then centrifuged at 10,000 g for 10 min to remove cellular debris. For the inoculation assay and the enzyme-linked immunosorbent assay (ELISA), the clarified saliva was used after filter sterilization through a 0.22 μm Acrodisc filter (Pall Corporation, Ann Arbor, MI). After filtration, they were pooled and stored at −20°C until used.

Preparation of immunoglobulin, amylase, and mucin

Lyophilized secretory Immunoglobulin A (sIgA) from human colostrum, α-amylase from human saliva, and mucin from bovine submaxillary glands (Sigma-Aldrich, St. Louis, MO) were mixed in PBS and adjusted to similar physiological concentrations as in human-saliva: 0.25, 0.4 and 2.7 mg/ml, respectively. These reagents were stored at −20°C until used.

Bacterial sampling and colony-forming unit (CFU) estimate

Bacterial inoculation, sampling and CFU estimates were performed using procedures and conditions described previously [31,34,35]. All oral streptococci were cultured in BHI broth overnight and then washed twice with sterile phosphate-buffered saline (PBS). Our previous study demonstrated that colony counts of *S. mutans* were significantly higher than that of other streptococci (i.e. *S. sanguis*, *S. sobrinus*, and *S. salivarius*) in mice that ingested 1% sucrose in water one day before inoculation [31]. Thus, mice were given drinking water containing 1% sucrose (less than the usual concentration in juice) one day prior to *S. mutans* inoculation to reproduce the early adherence of *S. mutans* in conditions resembling a natural state. Chlorhexidine (0.2%) soaked sterile cotton swabs were used to disinfect the oral cavities of the mice including the maxillary incisor teeth. The cavity was immediately washed with sterile PBS. Four or 6 mice were treated with 100 μl of human saliva or salivary components for 2.5 min with the aid of micropipette. Casein was used as a control as a non-salivary component for the treatment. Five min after treatment, mice were washed with 100 μl of PBS. *S. mutans* solutions were introduced to the oral cavities of all females at 4 months of age at a final concentration of 7×10^9 CFU in 250 μl of PBS during 2.5 min. Mice were separated into four groups based on the feeding conditions 24 h after inoculation. During the 24 h, one group was fed food with distilled water compared to another fed food with 1% sucrose-water; and the other set was food-deprived with 1% sucrose water or distilled water. Following inoculation, samples were collected from the labial surfaces of the maxillary incisor teeth with a sterile cotton ball and then dipped in 2 ml of PBS. To evaluate NOD/SCID.*e2f1*$^{-/-}$ mice as compared with previous results and to obtain stable data, samples collected from incisors were tested as parameters used in previous studies [31,35]. The samples in sterile PBS were sonicated using ultrasonic dispersion (power output, 60 W) for 10 s, and then poured onto Mitis-Salivarius agar plates containing 0.02 M bacitracin (MSB). CFUs were determined by counting rough-surface colonies on MSB plates after 48 h using anaerobic incubation at 37°C.

ELISA

To determine if sIgA reacts with *S. mutans* in vitro and if sIgA is absorbed on the tooth surface after treatment with human saliva, ELISA was performed with some modifications as described previously [33]. 96-well microtiter H-plates (Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with a culture of *S. mutans* or *A. naeslundii* (1 μl/ml) in Na_2CO_3 coating buffer at pH 9.6 and incubated at 4°C overnight. In the sandwich assay to

detect absorbed sIgA, we used 1/1,000 mouse anti-human immunoglobulin A antibody (Sigma-Aldrich, St. Louis, MO). The bacteria and antibody were diluted in Na_2CO_3 coating buffer at pH 9.6 and incubated at 4°C overnight. The plates were washed with PBS containing 0.1% (v/v) Tween 20 (PBST); and blocked with 1% (wt/vol) skim milk in PBST for 1 h at 37°C. Excess skim milk was removed by washing three times with PBST. To determine the presence of sIgA on the tooth surface, the tooth surface was swabbed using a sterile cotton ball after treatment with human saliva, and the swabbed ball was soaked in 2 ml coating buffer and shaken for 1 min. A 100 µl aliquot of 0.25 mg/ml sIgA, human saliva, or the soaked sample was added to the wells and the plates were incubated for 1 h at 37°C. The wells were washed three times with PBST; and further incubated for 1 h at 37°C with 100 µl 1/1,000 alkaline phosphatase conjugated goat anti-human immunoglobulin A antibodies (Zymed Laboratories, South San Francisco, CA). After three washings with PBST, the bound antibodies were detected after the addition of 50 µl of 3 mg/ml para-nitrophenyl phosphate as a substrate and incubated for 30 min at 37°C. Absorbance at 405 nm (A_{405}) was measured using a microplate reader (Multiskan Bichromatic; Laboratory Japan, Tokyo, Japan). The mean value for each sample was used to calculate the ELISA value: $Abs_{405} \times 100/t$ (t: time of reaction). Triplicate measurements were performed and means calculated with standard error.

Removal of *S. mutans*-specific sIgA

To determine if specific sIgA is employed for *S. mutans* colonization on the tooth surface, an absorption procedure was performed to remove specific antibody against *S. mutans*. Solutions of 1 mg/ml sIgA in PBS were absorbed with 0.5 mg (dry weight)/ml whole cells of lyophilized *S. mutans* UA159 at 37°C for 1 h and then overnight at 4°C. The mixture was centrifuged at 8,000 rpm for 10 min to remove *S. mutans*-IgA complex. Protein concentrations in the sIgA sample were measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratory, Hercules, CA) based on the method of Bradford and measured at 595 nm. The concentration of sIgA was adjusted to 0.25 mg/ml after the absorption procedure.

Inhibiting effects of FruA in biofilm formation with *S. mutans*

To determine if the animal model could be used for the analysis of inhibitors for colonization and biofilm formation of *S. mutans* on the tooth surface, fructanase (FruA), a candidate inhibitor for biofilm formation of *S. mutans* [36], was used in the *in vivo* assay. The inhibiting activity of FruA at 1.25 units/ml was assayed in 96 well microtiter plates coated with human saliva [36]. FruA at 1.25 units/ml was also added within a 1% sucrose solution in drinking water (DW). FruA does not digest sucrose at 20~25°C in 1% sucrose drinking water and does at 37°C in the oral cavity after mice drink the water [36]. After pre-treatment of sIgA following bacterial inoculation, all NOD/SCID.$e2f1^{-/-}$ mice were fed and supplied 1% sucrose water containing or not containing FruA. After 24 h inoculation, samples were collected and the CFU was counted as described above.

Statistical analyses

The CFU and ELISA data were expressed as means ± standard deviations. GraphPad Prism version 5.0 d for Mac OS X (GraphPad Software, San Diego, CA) was used to perform tests of significance. The statistical significance of differences between two groups was determined using the unpaired *t*-test. For comparison between multiple groups, one-way analysis of variance (ANOVA) and Tukey-Kramer tests were used. P-values less than 0.001, 0.01 or 0.05 were considered statistically significant using two-tailed comparisons. All experiments were repeated and analyzed independently.

Results

Colonization of *S. mutans* in mice treated with human saliva

Human saliva is thought to play a significant role in the attachment of *S. mutans* to the tooth surface. We evaluated human saliva in bacterial colonization of NOD/SCID wild type, NOD/SCID.$e2f1^{+/-}$ mice, and NOD/SCID.$e2f1^{-/-}$ mice. *S. mutans* colonization in each mouse was significantly increased at all time points after the inoculation when they were treated with human saliva (Fig. 1 A, B and C). Bacterial numbers on the tooth surfaces were significantly higher in NOD/SCID.$e2f1^{-/-}$ mice than those in NOD/SCID wild type or NOD/SCID.$e2f1^{+/-}$ mice after 90 and 120–180 min post inoculation (Fig. 1 D). Colony numbers of *S. mutans* gradually decreased from 30 min to 90 min; however, after the colonization phase, the CFU gradually increased from 90 to 180 min in human saliva-treated NOD/SCID.$e2f1^{-/-}$ mice; whereas the other mice did not show a difference comparing time points.

Effects of human saliva and salivary components in *S. mutans* colonization

To determine if salivary components induce colonization of *S. mutans* on the tooth surface using the *in vivo* model, α-amylase, mucin and sIgA, receptors for *S. mutans* adhesins, were used to treat teeth before bacterial inoculation. CFUs were lower within non-treated mice compared to NOD/SCID.$e2f1^{+/-}$ and $^{-/-}$ 18 mice treated with all components other than casein treatment (control; non-salivary component) in NOD/SCID.$e2f1^{+/+}$ mice (data not shown). NOD/SCID.$e2f1^{-/-}$ mice had a higher colonization than NOD/SCID.$e2f1^{+/-}$ and NOD/SCID.$e2f1^{+/+}$ in each pre-treatment using the salivary components (Fig. 2 A, B and C). Bacterial colonization on teeth treated with 0.25 mg/ml sIgA at physiological concentrations was increased significantly in NOD/SCID.$e2f1^{-/-}$ mice (13,992±6,423); however, there was no significant difference in treating with saliva compared to sIgA (Fig. 2 C). In NOD/SCID.$e2f1^{+/+}$ and NOD/SCID.$e2f1^{+/-}$ mice, treatment with 0.25 mg/ml sIgA did not show greater colonization (Fig. 2 A, B). Further, higher concentrations of sIgA (0.4 mg/ml) did not result in higher colonization by *S. mutans* in comparison with BSA and casein in NOD/SCID.$e2f1^{+/-}$ and NOD/SCID.$e2f1^{-/-}$ mice (Fig. 2 B and C). Treatment in NOD/SCID.$e2f1^{-/-}$ mice with mucin (at 0.4 and 2.7 mg/ml) or with BSA did not result in increased levels of *S. mutans* colonization; these pre-treatments yielded significantly lower CFU counts compared to treatment with 0.25 mg/ml sIgA and considerably higher counts compared to treatment with 0.4 mg/ml amylase. Treatment with amylase at 0.1 mg/ml showed significantly higher colonization than at 0.4 mg/ml in NOD/SCID.$e2f1^{+/-}$; whereas there was no significant difference using NOD/SCID.$e2f1^{-/-}$ mice.

SIgA was taken from human colostrum, and therefore may include various antibodies to pathogens. To confirm whether sIgA reacts with *S. mutans*, ELISA was performed using *S. mutans*-coated 96 well microtiter plates. *A. naeslunidii* was also used for coating as another oral bacterium. 0.25 mg/ml sIgA reacted strongly with *S. mutans* but not *A. naeslundii* (Fig. 3 A). The specificity of sIgA was observed by absorption of specific antibody to *S. mutans* in pre-incubation using *S. mutans* whole cells within sIgA. The absorbed sIgA was used for the ELISA assay and showed no significant

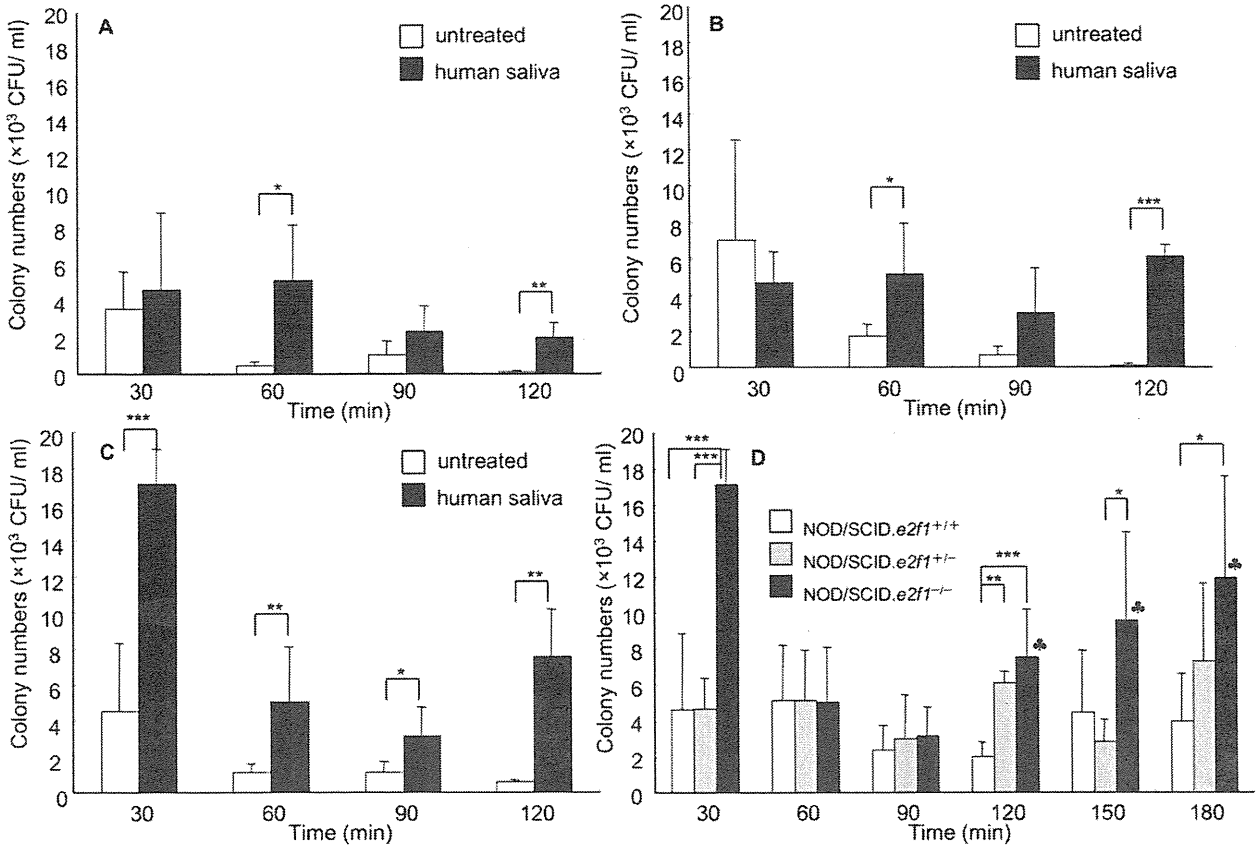

Figure 1. Colonization of *S. mutans* in human-saliva treated mice. Colony numbers of *S. mutans* in (A) NOD/SCID wild type, (B) NOD/SCID.*e2f1*$^{+/-}$, (C) NOD/SCID.*e2f1*$^{-/-}$ female mice, and 4 months of age pre-treated with and without human saliva prior to bacterial inoculation. Asterisks show significant differences (vs. untreated group, *$P<0.05$, ** $P<0.01$, *** $P<0.001$). (D) Time-course analysis of *S. mutans* colonization for each mouse strain pre-treated with human saliva prior to bacterial inoculation. Data were obtained from three independent experiments with 4 mice from each strain, and values are expressed as the means ± standard daviations (SDs) of the data (*$P<0.05$, ** $P<0.01$, *** $P<0.001$, represents significant differences vs. 90 min, $P<0.05$).
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reactivity to *S. mutans* (Fig. 3 A). Using human saliva, specific antibody to *S. mutans* was also observed using the ELISA assay (Fig. 3 A). The 0.25 mg/ml absorbed sIgA was used for the colonization assay in NOD/SCID wild type, NOD/SCID.*e2f1*$^{+/-}$ and NOD/SCID.*e2f1*$^{-/-}$ mice, and the effect of absorbed sIgA was compared with 0.25 mg/ml non-absorbed sIgA in all mice. The absorbed sIgA did not increase colonization of *S. mutans* in comparison with non-absorbed sIgA at 120 min after inoculation of *S. mutans* (Fig. 3 B). Therefore, increased colonization of *S. mutans* was dependent on specific antibody to *S. mutans* in sIgA and human saliva using this animal model. To determine whether sIgA remained on the tooth surface after treatment with human saliva, the surface was swabbed using a sterilized cotton ball at 120 min after the treatment in mice; and sIgA in the swabbed sample was measured using ELISA. The level of human-IgA that remained on the teeth for 120 min was significantly higher in NOD/SCID.*e2f1*$^{-/-}$ mice as compared to the other two strains (Fig. 3 C). This shows that specific sIgA antibody to *S. mutans* remains on the tooth surface after treatments with sIgA and human saliva in mice having decreased saliva and lack of IgA and IgG, the NOD/SCID.*e2f1*$^{-/-}$ mice. To determine whether a lack of IgA, by inserting the SCID type in NOD.*e2f1*$^{-/-}$ mice, promoted the colonization of *S. mutans*, the parent strain (NOD.*e2f1*$^{-/-}$ mice) and previous the parent strain (NOD mice) to NOD.*e2f1*$^{-/-}$ mice were used for the colonization assay after pre-treatment with 0.25 mg/ml sIgA and compared with NOD/SCID.*e2f1*$^{-/-}$ mice. We found that the colonization at 120 min after inoculation was significantly lower in NOD and NOD.*e2f1*$^{-/-}$ mice than NOD/SCID.*e2f1*$^{-/-}$ mice (Fig. 3 D). Therefore, lack of IgA and decreased saliva allowed specific IgA to remain on the tooth surface and to promote colonization of *S. mutans* in NOD/SCID.*e2f1*$^{-/-}$ mice.

Synergistic effects of sucrose water and diet, and human saliva on *S. mutans* long-term colonization

Long-term colonization is necessary in a mouse model to study several agents for the prevention to oral diseases. We observed that after inoculation, the colonization of *S. mutans* was slight at 24 hours in NOD/SCID, NOD/SCID.*e2f1* $^{+/-}$ and NOD/SCID.*e2f1*$^{-/-}$ mice pre-treated with human saliva (Fig. 4 A). Drinking water and diet including sucrose helped biofilm formation in other studies [14,31]. A low concentration of 1% sucrose water was selected and supplied as drinking water with the usual animal diet for mice to establish an animal model that avoided high sucrose concentration-dependent colonization. The significant colonization was not observed in only the 1% sucrose water group as compared to that in non-sucrose water and non-diet group (Fig. 4 A, B). However, the group supplied with the combination of 1% sucrose-water and diet showed the most CFU/ml of *S. mutans*; colony numbers in NOD/SCID.*e2f1*$^{-/-}$