beginning of the SPT phase for a cohort study (Fig. 1B). Patients exhibiting IgG antibody titer levels significantly ($>2\sigma$) above the average among healthy volunteers are defined as having high-level serum IgG antibody titer against periodonopathic bacteria. Significant differences of periodontitis recurrence ratio between each group were analyzed by Pearson's χ^2 test.

For statistical analysis, computer software Statview 5.0 (Abacus Concepts, Inc., Berkeley, CA) was used.

RESULTS

Clinical Findings of Patients Before SPT Phase

Chronic periodontitis of all patients were treated by intensive periodontal treatment. The healing was evaluated by trained dentists using routine periodontal examination methods (periodontal pocket depth, BOP, and X-ray). A total of 139 patients during SPT phase were analyzed for case–control study (Stable group: 112, Recurrence group: 27). Clinical findings of patients before SPT phase are summarized in Table 1. There were no significant differences between the stable and recurrence group in the score of their PCR, BOP, and even averaged probing pocket depth. On the other hand, there were significant differences between the stable and recurrence groups in their age and number of teeth (age, $P = 0.026$: number of teeth, $P = 0.025$: Mann–Whitney U-test).

Statistical Differences Between the Stable and Recurrence Group in Serum IgG Antibody Titer Before Transition to SPT Phase

In 12 strains from 8 bacterial species, average of serum IgG antibody titer against all of periodontal bacteria before transition to SPT phase in the recurrence group was higher than that of the stable group (Fig. 2). Especially, the levels of serum IgG antibody titer to several periodontal bacteria were statistically higher in the recurrence group than that of the stable group before transition to SPT phase (*A. actinomycetemcomitans* Y4, $P = 0.020$: *E. corrodens* ATCC1073, $P = 0.040$: *P. gingivalis* SU63, $P = 0.020$: *C. rectus* ATCC33238, $P = 0.025$: Mann–Whitney U-test). The serum IgG antibody titer against *T. denticola* ATCC35405 was also clearly higher in the recurrence group than in the stable group ($P = 0.081$: Mann–Whitney U-test) before transition to SPT phase.

Statistical Differences Between the High and Normal Serum IgG Titer Group in Periodontitis Recurrence

In a cohort study, the patients were categorized into two groups according to their serum IgG antibody titer levels associated with the eight known periodontal bacteria. In the "normal" group, the level of serum IgG antibody titer was observed to be lower than 1.0 against each type of bacteria at the beginning of the SPT phase. In the "high" group, the level of serum IgG antibody titer exceeds 1.0 against periodontal bacteria. As shown in Table 2, importantly, we found that there were no significant differences between the Normal and High serum IgG antibody titer group in all clinical findings. From these clinical data, we confirmed to become healthy clinically in both groups by active periodontal treatment. Furthermore, we observed the tendency that the recurrence ratio of the high serum IgG titer group was higher than that of the normal group (Normal group: 14.9–19.0 %, High group: 20.5–36.8 %). Especially, the recurrence ratio of the high IgG titer group to three obligate anaerobic bacteria was statistically higher than that of the normal titer group (*P. intermedia* ATCC25611, $P = 0.021$; *T. denticola* ATCC35405, $P = 0.039$; *C. rectus* ATCC33238, $P = 0.048$: Pearson's χ^2 test). In addition, the recurrence ratio of the high titer group against *P. gingivalis* SU63 was higher than that of the normal titer group, although there was no statistical difference ($P = 0.083$: Pearson's χ^2 test). Furthermore, we examined the combined recurrence ratio in high IgG antibody titer against 12 periodontal bacteria, and the periodontitis recurrence ratio of the high titer group was

TABLE 1. Clinical Findings at the Beginning of SPT Phase

	Stable group ($N = 112$)	Recurrence group ($N = 27$)	P-value
Age (yr)	60.2±10.6	67.0±8 1	0.026*
Number of teeth	22.0±6.3	17.2±82	0.025*
PCR (%)	21.5±15.1	22.4±13.0	0.775
BOP (%)	11.3±11.0	13.9±87	0.224
Pocket depth (mm)	2.30±0.3	2.50±0 5	0.158
SPT period (month)	48.9±12.4	51.8±12.5	0.362

Clinical findings excluding SPT period were examined at the beginning of SPT phase. PCR, Plaque control record; BOP, Bleeding on probing. *Significant difference ($P<0.05$, Mann–Whitney U-test) between stable and recurrence group. Values represent the mean±standard deviation (SD).

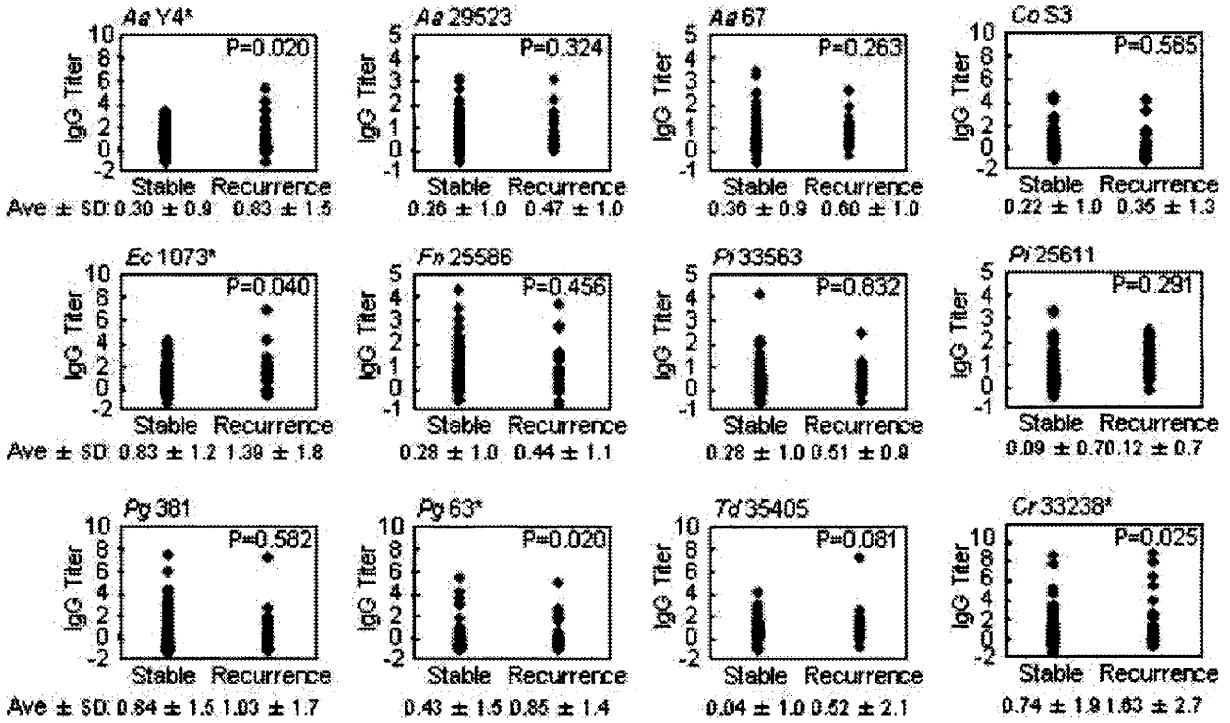

Fig. 2. The levels of serum IgG antibody titer against 12 periodontal bacteria. The significant differences between "Stable" and "Recurrence" group were analyzed using the Mann–Whitney *U*-test. Each dot represents an individual data tested by ELISA assay. The *Y*-axis (IgG Titer) in each panel denotes the value determined as (serum IgG titer tested by ELISA)−(mean titer calculated using that of healthy subjects)/(2 SD calculated using that of healthy subjects) as described in Materials and Methods section. Ave, average of IgG Titer: each data have calculated and shown as average±SD. $^*P<0.05$. *Aa, A. actinomycetemcomitans*; *Co, C. ochracea*; *Ec, E. corrodens*; *Fn, F. nucleatum*; *Pi, P. intermedia*; *Pg, P. gingivalis*; *Td, T. denticola*; *Cr, C. rectus*.

greater than that of the normal titer group (High titer group: 21.6 % ($N=97$), Normal titer group: 14.3% ($N=42$), $P=0.339$, Pearson's χ^2 test).

DISCUSSION

Periodontal disease is a common chronic infection caused by Gram-negative bacteria such as *P. gingivalis* and *P. intermedia* (1). Recurrence of periodontitis may lead to poor oral health, and result in tooth loss. Therefore, in order to prevent the recurrence of the disease after periodontal treatment, it is important to establish the efficient methods for patients. Recently, epidemiological research provides strong evidence that periodontitis is a risk factor for systemic diseases such as cardiovascular disease (5,6). A number of studies have reported that periodontal infection would be a risk factor for progression of myocardial infarction and stroke (15,16). Therefore, persistent low-grade infection by chronic periodontitis is also a focus for physicians.

This study is a part of our ongoing efforts to elucidate the clinical usefulness of serum IgG antibody titer to periodontal bacteria. In general, it is well recognized that periodontitis is a multifactorial disease (17–19). For example, a young patient developing periodontitis might be most likely a carrier of one or more genetic factors. Patients may also have one or more chronic systemic diseases associated with an increased risk for periodontitis. Therefore, it is difficult to identify the factors contributing to the onset, progression, and the recurrence of periodontitis following periodontal therapy.

Good control of supragingival plaque is important to prevent the periodontitis recurrence in SPT phase, after intensive periodontal treatment. However, our results have shown that the predictive value of routine periodontal parameters (PCR, BOP, and pocket probing depth) is relatively low (Table 1). Periodontal examinations we performed routinely did not provide clear predictions for the recurrence of periodontitis. This is not unexpected because routine periodontal examinations such as BOP and pocket probing depth primarily indicate the past reaction to inflamed periodontal tissue. As shown in Table 1, among the factors relating to the periodontitis recurrence during SPT phase, we found age of patients is one of the risk factors in the recurrence. With age, metabolism, restoration ability, and preventive ability of

TABLE 2. Clinical Findings After Periodontitis Treatment and Reccurrence Ratio During SPT

	Strains	Examination	Normal laG titer	High IgG titer	*P*-value
Facultative anaerobic	*Aa* Y4	Patients number	104	35	
		Age (yr)	60.1±10.7	64.0±9.2	0.16
		Number of teeth	21.8	20	0.17
		PCR (%)	21.3	25.7	0.47
		BOP (%)	11.7	14.4	0.51
		Pocket depth (mm)	2.32	2.29	0.66
		Serum IgG Ab. Titer	0.079	2.51	<0.0001
		Recurrence ratio (%)	17.3	25.7	0.28
	Aa ATCC29523	Patients number	107	35	
		Age (yr)	61.2±10.6	61.5±10.3	0.92
		Number of teeth	22.2	19.2	0.085
		PCR (%)	22.7	21.6	0.54
		BOP (%)	12.4	12.4	0.79
		Pocket depth (mm)	2.28	2.41	0.39
		Serum IgG Ab. Titer	0.11	2.69	<0.0001
		Recurrence ratio (%)	16.8	28.1	0.16
	Ec FDC1073	Patients number	82	57	
		Age (yr)	60.8±10.4	61.6±10.5	0.69
		Number of teeth	22.1	20.2	0.064
		PCR (%)	23.1	21.6	0.41
		BOP (%)	12.4	12.2	0.63
		Pocket depth (mm)	2.31	2.33	0.89
		Serum IgG Ab. Titer	0.11	2.64	<0.0001
		Recurrence ratio (%)	15.9	24.6	0.21
Obligate anaerobic	*Pi* ATCC25611*	Patients number	115	24	
		Age (yr)	61.3±10.1	61.1±12.5	0.93
		Number of teeth	21.6	20.2	0.49
		PCR (%)	22.3	23.3	0.84
		BOP (%)	12.2	13.7	0.51
		Pocket depth (mm)	2.31	2.39	0.24
		Serum IgG Ab. Titer	0.02	2.07	<0.0001
		Recurrence ratio (%)	15.8	36.1	0.021
	Pg FDC381	Patients number	100	39	
		Age (yr)	61.7±10.5	60.1±10.5	0.56
		Number of teeth	21.8	20.2	0.43
		PCR (%)	23.1	21.3	0.39
		BOP (%)	12.4	12.4	0.99
		Pocket depth (mm)	2.29	2.38	0.59
		Serum IgG Ab. Titer	0.14	3.14	<0.0001
		Recurrence ratio (%)	19.1	20.5	0.84
	Pg SU63	Patients number	113	26	
		Age (yr)	61.8±10.6	57.8±9.3	0.18
		Number of teeth	21.2	22.1	0.99
		PCR (%)	24.2	12.4	0.29
		BOP (%)	13.1	9.1	0.15
		Pocket depth (mm)	2.31	2.33	0.95
		Serum IgG Ab. Titer	0.004	3.13	<0.0001
		Recurrence ratio (%)	16.8	36.1	0.083
	Td ATCC35405*	Patients number	120	19	
		Age (yr)	61.1±10.2	61.3±12.3	0.88
		Number of teeth	21.8	18.8	0.14
		PCR (%)	23.3	17.8	0.24
		BOP (%)	12.7	10.4	0.67
		Pocket depth (mm)	2.33	2.23	0.22
		Serum IgG Ab. Titer	0.21	2.31	<0.0001
		Recurrence ratio (%)	16.7	36.8	0.039
	Cr ATCC33238*	Patients number	100	39	
		Age (yr)	61.5±10.1	60.6±11.4	0.79
		Number of teeth	22.1	19.9	0.22
		PCR (%)	22.1	23.3	0.76

TABLE 2. Continued

Strains	Examination	Normal laG titer	High IgG titer	*P*-value
	BOP (%)	11.8	13.6	0.65
	Pocket depth (mm)	2.26	2.42	0.13
	Serum IgG Ab. Titer	0.02	3.67	<0.0001
	Recurrence ratio (%)	14.9	29.7	0.048

Data were analyzed by Mann–Whitney *U*-test for clinical findings and Pearson's χ^2 test for Recurrence ratio between "Normal" and "High" IgG titer group. *, $P<0.05$: The recurrence ratio in "High" IgG Titer group is significantly higher. *Aa, A. actinomycetemcomitans; Ec, E. corrodens; Pi, P. intermedia; Pg, P. gingivalis; Td, T. denticola; Cr, C. rectus.*

periodontal tissue cells are reduced irreversibly. Therefore, the risk of periodontitis recurrence might increase with the age of patients indirectly.

There have been reports that measurement of serum IgG antibody titer was useful for diagnosing periodontitis or judging the treatment effects (15). However, during the SPT phase following active periodontal treatment, the usefulness of the levels of serum IgG antibody titer was still unknown. We have proposed a new insight for the prognosis of periodontitis recurrence during SPT phase using serum IgG antibody titer. In this study, we analyzed the usefulness of the levels of serum IgG antibody titer in predicting the recurrence of periodontitis during SPT phase by multiple classification analysis. We used sonic extracts of whole bacterial cells as antigens for ELISA. As the bacterial antigens include various components, mainly protein, lipopolysaccharide (LPS), and DNA, the serum IgG antibody titer against periodontal bacteria reflects total results of antibody responses (8).

Periodontitis is a bacterial infectious disease (17). The humoral responses against bacteria are largely different among individuals. The immunological response against specific bacteria should be clinically useful for evaluating the risk of periodontitis recurrence. Figure 2 shows the levels of serum IgG antibody titer against 12 periodontal bacteria before transition to SPT phase in the stable and recurrence group. Interestingly, although the levels of serum IgG antibody titer against all periodontal bacteria were variable, we found that the serum IgG antibody titer against several bacteria (*A. actinomycetemcomitans* Y4, *E. corrodens* ATCC1073, *P. gingivalis* SU63, and *C. rectus* ATCC33238) was significantly higher within the recurrence group than the stable group when in transition to SPT phase. These findings indicate that serum IgG antibody titer might be useful clinically as a diagnostic marker of periodontitis recurrence during SPT phase.

From another viewpoint, we examined the differences of the periodontitis recurrence ratio between the high and normal serum IgG antibody titer group when transition to SPT as a companion study. Interestingly, we observed the tendency that the recurrence ratio of the high serum IgG titer group was higher than that of the normal group as shown in Table 2. Especially, we found the recurrence ratio of the high titer group against several periodontal bacteria (*P. intermedia* ATCC25611, *T. denticola* ATCC35405, and *C. rectus* ATCC33238) was statistically higher than that of the normal titer group. Furthermore, we examined the combined recurrence ratio in high IgG antibody titer against 12 periodontal bacteria. Interestingly, we found that the periodontitis recurrence ratio of the high titer group was greater than that of the normal titer group. The combined periodontal bacteria might provide an effective clinical prognosis of periodontitis recurrence. Our findings indicate that the serum IgG antibody titer might be useful as a predicting marker of periodontitis recurrence during SPT phase. Also, Tolo et al. reported that the level of serum IgG antibody titer against *P. gingivalis* increases before absorption of alveolar bone, and could predict the progression of periodontitis (20). This report supports our concept.

According to recent studies, chronic periodontitis, persistent low-grade infection of Gram-negative bacteria, is associated with increased atherosclerosis, heart disease, diabetes mellitus, and other systemic diseases through the blood stream (4,5,21). So poor oral health may have profound effect on general health; therefore, it is important to prevent the recurrence of periodontitis for health promotion practice.

We believe that SPT is effective for preventing the recurrence of periodontitis. In this study, we wanted to find the primary risk factors of periodontitis recurrence in patients after periodontal treatment. From multiple classification analysis on clinical findings and serum IgG antibody titers before transition to SPT phase, we elucidated the predictive markers for the recurrence of periodontitis in view of humoral immune responses to periodontal infection. We propose the attention should be focused on the levels of serum IgG antibody to periodontal bacteria when transition to SPT phase. Our findings show that elevated serum IgG antibody titer is an important marker to predict the periodontitis recurrence during the transition to SPT phase.

ACKNOWLEDGMENTS

We greatly thank Scott Messenger at NASA Johnson Space Center for the revision of the manuscript and for encouragement in our research. This study was supported by Grant-in-Aid for Scientific Research (A) (No. 18209061) from the Japan Society for the Promotion of Science, and by Health and Labour Sciences Research Grants (Comprehensive Research on Aging and Health, H19-Choju-008) from the Ministry of Health, Labour and Welfare of Japan.

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Letters in
Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

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

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

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2010/1427: received 12 August 2009, revised 19 December 2009 and accepted 14 January 2010

doi:10.1111/j.1472-765X.2010.02806.x

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′-aggttctttttatcttcggtta-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*

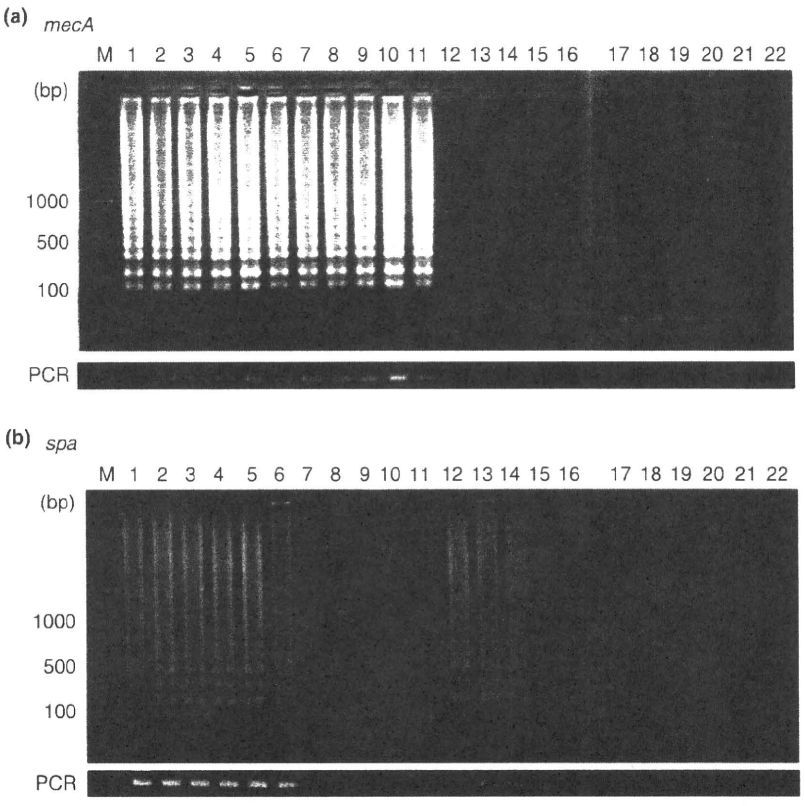

Figure 1 Specificity tests of the LAMP for *mecA* (a) and *spa* (b). All bacterial species prepared in the current study were tested, and the representative results were shown. The *mecA* and *spa* were simultaneously detected by PCR, and the amplicons were shown below the LAMP results. Lane M: DNA size 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).

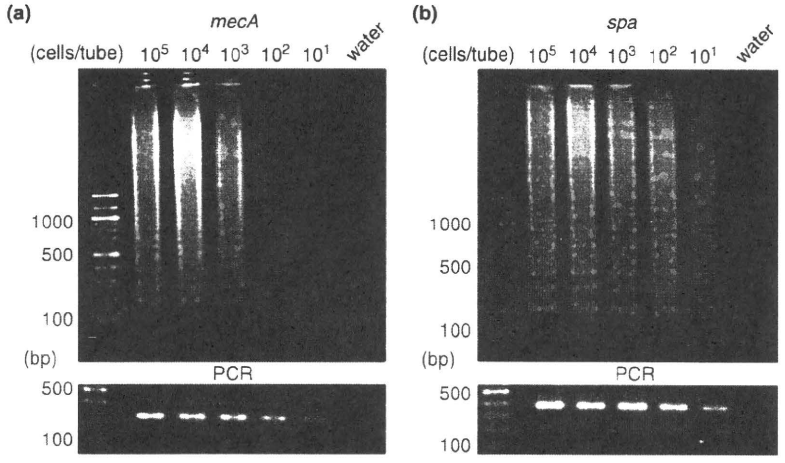

Figure 2 Detection limits of the loop-mediated isothermal amplification (LAMP) for *mecA* (a) and *spa* (b). DNAs were extracted from serial diluted cells (10–10^5 cells) of Methicillin-resistant *Staphylococcus aureus* (MRSA) (NCTC 10442) and were used as templates. The detection limits of the conventional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

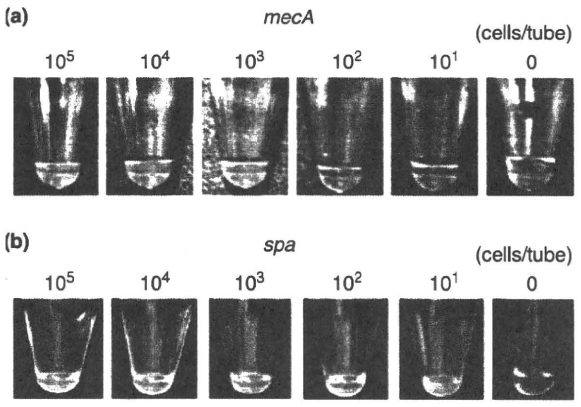

Figure 3 Naked-eye inspection of the LAMP for *mecA* (a) and *spa* (b). The original orange colour of SYBR green I turned to be green in the positive reaction mixture.

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

LAMP assay	PCR assay		Percent agreement (kappa value)	Sensitivity	Specificity	PPV*	NPV†
	Positive	Negative					
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*.

Acknowledgements

We thank Dr T. Ito (Department of Bacteriology, Juntendo University) for providing MRSA strains. This study was supported by Grants-in-Aid for Scientific Research (B17390502 to S.K. and C21592624 to H.M.) from the Japan Society for the Promotion of Science.

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Support Care Cancer (2011) 19:303–307
DOI 10.1007/s00520-010-1002-y

SHORT COMMUNICATION

Progress of oral care and reduction of oral mucositis—a pilot study in a hematopoietic stem cell transplantation ward

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Received: 20 January 2010 / Accepted: 31 August 2010 / Published online: 15 September 2010

Abstract
Purpose Oral mucositis is a common symptomatic complication associated with hematopoietic stem cell transplantation (HCT). We use simple strategies aimed to reduce oral mucositis by keeping the oral cavity clean and moist. Here, we report on the progress of oral care and the changes in the degree of oral mucositis. The purpose of this pilot study is to evaluate the effects of our strategies on the prevalence and the severity of oral mucositis.
Methods Fifty-three consecutive patients from 2003 to 2006 administered with conventional allogeneic HCT were enrolled in this study. The degree of oral mucositis was evaluated daily in all patients. Our oral care program was divided into two periods: "examination and trial period (2003 and 2004)" and "intensive oral care period (2005 and 2006)." In the latter, an oral care regimen was carried out systematically by a multidisciplinary team.
Results Using our oral care strategies, the prevalence of ulcerative oral mucositis was decreased significantly. The rate was reduced from 76% (10 of 13) of patients with ulcerative oral mucositis in 2003 to only 20% (3 of 15) in 2006.
Conclusions Our pilot study suggests that oral mucositis in HCT patients can be alleviated by simple strategies aimed at keeping the oral cavity clean and moist.

Keywords Oral care · Supportive care · Oral mucositis · Hematopoietic stem cell transplantation

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Introduction

Oral mucositis is one of the most common symptomatic complications associated with high-dose chemotherapy, especially hematopoietic stem cell transplantation (HCT) [1, 2]. Severe mucositis is associated with not only intolerable pain but also the risk of systemic infection. Oral mucositis is a significant cause of suffering and morbidity in patients receiving myeloablative chemotherapy [3]. Effective interventions to alleviate this complication are needed [3].

Keeping the oral cavity clean is one of the important interventions because this prevents both mucositis itself and infection associated with oral mucositis. The Multinational Association for Supportive Care/International Society of Oral Oncology mucositis guidelines [4] and the National Cancer Center Network task force report [5] both recommend good oral hygiene in these patients.

Keeping the oral cavity moist may also be important. Oral dryness is caused by high-dose chemotherapy and total-body irradiation (TBI) performed as part of the conditioning regimen for HCT. Oral dryness not only results in discomfort

but may also exacerbate oral mucositis. We have often seen the development of ulcerative mucositis on dry mucosa in contact with dry teeth clinically. One of the reasons may be that saliva is necessary to maintain oral mucosal health. Additionally, moisture in oral cavity may moderate irritation caused by mechanical contact between the teeth and oral mucosa.

We began attempts to implement oral care in our ward from 2003. Our strategy includes a multidisciplinary approach prior to and during cancer treatment aimed at reducing the oral microbial load and keeping the oral cavity moist. Here, we describe the effect of intensive oral care on the degree of oral mucositis in HCT recipients.

Materials and methods

Patients

Fifty-three consecutive patients administered conventional allogeneic HCT at Okayama University Hospital of Medicine and Dentistry between April 2003 and March 2007 (23 men, 30 women; mean age±SD, 34.3±11.8 y) were enrolled in this study. Patients administered autologous and reduced-intensity HCT (RIST) were excluded. Numbers of patients and diseases according to year are shown in Table 1.

The Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences approved this study.

HCT conditioning regimens

Most patients with related or unrelated donors received TBI at a dose of 12 Gy in six fractions followed by cyclophosphamide (CY) at a dose of 60 mg/kg once daily for 2 days. Alternatively, patients received a combination of busulphan (BU; 4 mg/kg/day×4 days) and CY (60 mg/kg/day×2 days). Patients with unrelated cord blood donors were treated with TBI at 12 Gy, CY (60 mg/kg/day×2 days) and cytarabine (Ara-C; 6 g/m^2/day×2 days). Numbers of patients, sources of hematopoietic stem cells, and HCT protocols (conditioning regimen) according to year are shown in Tables 2 and 3.

General infection control

Fluoroquinolone for prophylaxis against bacterial infection and fluconazole for prophylaxis against fungal infection were administered orally. Prophylaxis against herpes virus infection with acyclovir was also given. Neutropenic fever was managed according to the guidelines of Hughes et al. [6].

Assessment of oral mucositis

The severity of oral mucositis in patients undergoing HCT was evaluated daily according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0 [7]. The criteria for oral mucositis were as follows:

- Grade 1: Erythema of the mucosa
- Grade 2: Patchy ulcerations or pseudomembranes
- Grade 3: Confluent ulcerations or pseudomembranes; bleeding in response to minor trauma
- Grade 4: Tissue necrosis; significant spontaneous bleeding; life-threatening consequences
- Grade 5: Death

Assessments were performed as part of daily nursing care by nurses who were trained by dentists and dental hygienists. The consistency of these assessments was checked by the dental team at least once per week.

Progress of our oral care regimen

Implementation of our oral care program was divided into two periods: "examination and trial period (2003 and 2004)" and "intensive oral care period (2005 and 2006)." Throughout this study period, the core oral care providers consisting of an experienced dentist, dental hygienists, and nurses were the same.

Table 1 Diseases of patients

Diseases	Year				Total
	2003	2004	2005	2006	
Acute myelogenous leukemia	5	4	3	4	16
Acute lymphoblastic leukemia	5	3	1	2	11
Chronic myelogenous leukemia	1	0	1	0	2
Malignant lymphoma	1	4	6	4	15
Aplastic anemia	1	0	1	0	2
Myelodysplastic syndromes	0	0	2	5	7
Total	13	11	14	15	53

Table 2 Source of hematopoietic stem cells

Source	Year				Total
	2003	2004	2005	2006	
Related donors	7	4	2	6	19
Unrelated donors (without cord blood donors)	4	7	7	4	22
Unrelated cord blood donors	2	0	5	5	12
Total	13	11	14	15	53

Examination and trial period (2003 and 2004)

We provided oral care interventions when oral mucositis developed clinically in HCT patients. On the other hand, there was no consensus within our ward regarding the precise method of oral care, and sometimes some points were missed.

Intensive oral care period (2005 and 2006)

We provided preventive oral care interventions, keeping the oral cavity clean and moist. The core oral care providers educated all ward staff members including new personnel. The oral care regimen included:

1. All subjects were referred to dentists with experience in treating medically compromised patients, and necessary dental treatment aimed at reducing preexistent oral infection, and the oral microbial load was completed as much as possible before HCT.
2. All subjects were instructed regarding self management including performing meticulous oral hygiene geared to their individual needs. Staff members, including nurses and dental professionals performed oral hygiene measures to patients in poor general condition. In patients with severe mucositis who could not tolerate tooth brushing, dental and mucosal debris was gently removed using saline-drenched gauzes, aimed at keeping the oral cavity as clean as possible.
3. Oral rinsing with saline was performed every 3 h during daytime. In addition, patients used a commercial saliva substitute, Oralbalance®, when they experienced oral dryness. Oral rinsing with chlorhexidine is not recommended in Japan. Oral rinsing with amphotericin B was indicated only when fungi were detected on the oral mucosa.

Table 3 Conditioning regimen of HCT

Conditioning regimens	Year				Total
	2003	2004	2005	2006	
With TBI					
CY/TBI	6	5	6	9	26
l-PAM/TBI	2	2	3	2	9
CA/TBI	1	0	0	0	1
CA/CY/TBI	0	1	3	2	6
CY/TBI/ATG	0	0	1	0	1
Without TBI					
BU/CY	3	3	1	1	8
CY/ALG	1	0	0	0	1
Flu/BU	0	0	0	1	1
Total	13	11	14	15	53

TBI Total-body irradiation, *CY* cyclophosphamide, *L-PAM* melphalan, *CA* cytarabine, *ATG* anti-thymocyte globulin, *BU* busulfan, *ALG* anti-lymphocyte globulin, *Flu* fludarabine

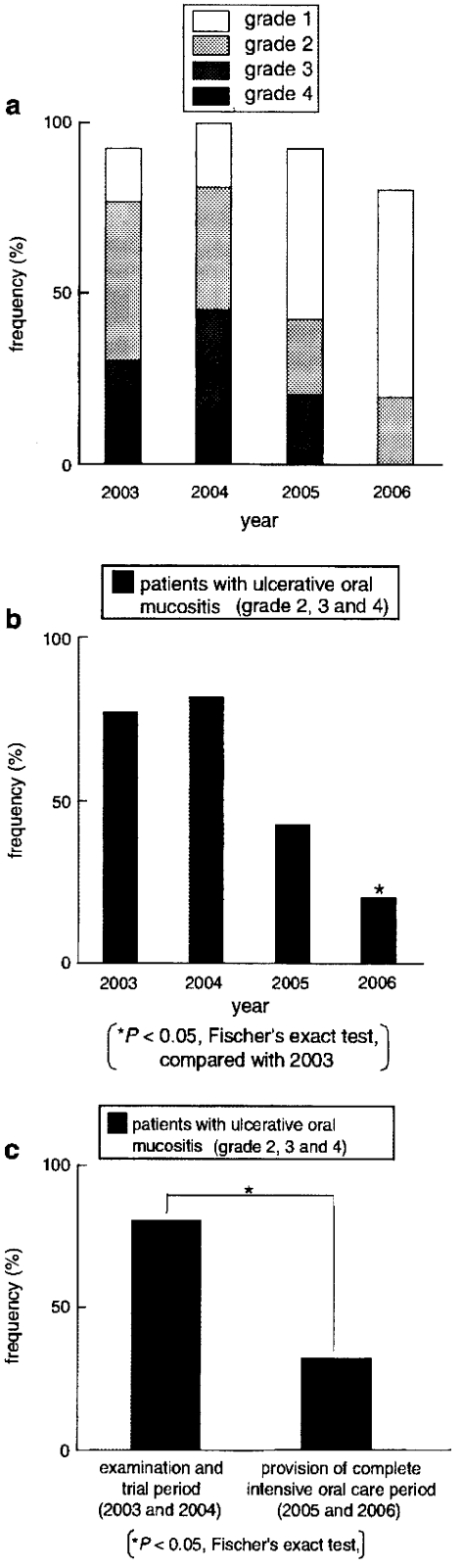
Statistical analysis

The frequencies of patients with oral ulcerative mucositis (NCI-CTCAE version 3.0≥2) during transplantation period for each year were analyzed statistically with Fischer's exact test. Mucositis frequencies of 2004, 2005, and 2006 were compared with that of 2003, and the period 2003–2004 and 2005–2006 were compared. P values were calculated using StatFlex statistical software (Artech, Osaka, Japan).

Results

Progress of oral care and oral mucositis

The frequencies of all grades of mucositis by year are shown in Fig. 1a. Subjects were categorized as follows: non-oral ulcer < Grade 1; oral ulcer carrier > Grade 2, since mucositis ≥ Grade 2 suggests disruption of the oral mucosal membrane barrier and formation of an infection route (Fig. 1b). With progress of oral care, the frequency of patients with ulcerative oral mucositis decreased significantly ($P \leq 0.05$, Fischer's exact test), whereas there were no significant changes relative to diseases or conditioning regimens (Tables 1 and 2); mucositis rate was reduced from 76% (10 of 13) of patients with ulcerative oral mucositis in 2003 to only 20% (3 of 15) in 2006. When the historical control group (2003+2004) was compared with the intensive oral care regimen group (2005+2006), a significant reduction in ulcerative mucositis was also observed (Fig. 1c; $P \leq 0.05$, Fischer's exact test).



◀**Fig. 1** Frequencies of oral ulcerative mucositis by year. **a** Frequencies of all grades of mucositis by year. **b** Frequencies of patients with ulcerative oral mucositis (grade > 2). Numbers of patients with ulcerative oral mucositis according to the year of their HCT were as follows: 10 of 13 in 2003; 9 of 11 in 2004; 6 of 14 in 2005; 3 of 15 in 2006. **c** The historical control group (2003+2004) was compared with the intensive oral care regimen group (2005+2006). A significant reduction of ulcerative mucositis was observed

Discussion

Our oral care strategy aimed at keeping the oral cavity clean and moist reduced the degree of ulcerative oral mucositis in our ward. Borowski et al. [8] reported the superiority of intensive oral care in patients with and without TBI and in patients with good or poor oral hygiene; the observed risk of mucositis was reduced by 70% in each of these four subgroups in their study. Our results were very similar to those reported in this study. The ulcerative mucositis rate in our study was reduced from 76% (10 of 13) of patients in 2003 to only 20% (3 of 15) in 2006. Therefore, the rate of ulcerative mucositis in 2003 was reduced by 73.7% in 2006 by our intensive oral care regimen (Fig. 1b).

The Multinational Association for Supportive Care in Cancer/International Society of Oral Oncology mucositis guidelines recommend systematic oral care with brushing, flossing, bland rinses, and moisturizers [4]. This guideline recommends a multidisciplinary approach to oral care including nurses, physicians, dentists, dental hygienists, dieticians, pharmacists, and others, when relevant. Furthermore, dental examination and treatment are considered important prior to the start of cancer therapy [4]. The present pilot study supports these recommendations. Our oral care regimen included application of Oralbalance®, which has been shown to have an antimicrobial effect [9]. However, the use of additional antimicrobial agents may be indicated in patients who cannot continue tooth brushing. In our regimen, we used wet gauzes to clean the oral cavity in these patients, but this has been shown to be ineffective to remove dental plaque [10].

The shifts in some of the diagnoses (Table 1) and associated treatment regimens between the two periods evaluated may have had an impact on the outcomes. A prospective intervention study, including large numbers of subjects and controls, may provide more detailed information on optimal oral care measures and may demonstrate the significance of oral care in HCT patients to reduce oral mucositis and related outcomes including pain, fever and infection, length of hospital stay, and costs.

In conclusion, our results suggest that oral mucositis in HCT patients can be alleviated by intensive multidisciplinary oral care starting prior to HCT and aimed at keeping the oral cavity clean and moist in the immediate post-transplant phase.

Acknowledgments This study was partially conducted as the education curriculum, Practice in Dental Sciences, in Okayama University Dental School. We thank Ms. Yuka Oka, a student at Okayama University Dental School, for data analysis.

This study was partly supported by a Grant-in-Aid for Young Scientists (Start-up #20890138 and B #22791836 to YS) from the Japan Society for the Promotion of Science and an unrestricted grant from the Scholarship Fund donated by the T&K Corporation, Tokyo, Japan (to ST). T&K Corporation is the exclusive agent of Biotene® products, including Oralbalance® (Laclede, Rancho Dominguez, CA), in Japan. Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences approved this private scholarship fund.

Conflicts of Interest None

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Support Care Cancer
DOI 10.1007/s00520-010-0923-9

ORIGINAL ARTICLE

Bacterial substitution of coagulase-negative staphylococci for streptococci on the oral mucosa after hematopoietic cell transplantation

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Received: 5 December 2009 / Accepted: 20 May 2010

Abstract
Purpose Coagulase-negative staphylococci (CoNS) are frequently isolated from blood cultures of hematopoietic cell transplantation (HCT) patients. Generally, the use of central venous catheters is recognized as a significant risk factor for CoNS infection, while the impact of CoNS infection from oral ulcerative mucositis, which occurs frequently in HCT, may be underestimated. Here, we examined the bacteria on the buccal mucosa after HCT.
Methods Sixty-one patients were examined for bacteria on the buccal mucosa routinely once a week from 1 week before to 3 weeks after allogeneic HCT. Subjects were divided into groups with short and long periods of antibiotic use, and differences in bacterial substitution were evaluated. The relationships between type of HCT (conventional HCT or RIST) and bacterial substitution were also evaluated.
Results The changes in detection frequencies of CoNS and α-streptococci from before to 3 weeks after HCT were significant ($P<0.05$, χ^2 test): 14.5–53.3% and 92.7–53.1%, respectively. Significant bacterial substitution of CoNS for streptococci was observed in the long-term antibiotic use group ($P<0.05$, χ^2 test), but also occurred in cases with short-term or no antibiotic use. No relationships between type of HCT (conventional HCT or RIST) were observed.
Conclusion Bacterial substitution of CoNS for streptococci occurred frequently on the buccal mucosa after HCT. In addition to antibiotic use, environmental factors may be involved in bacterial substitution. It is important to consider the presence of oral mucositis in CoNS infection after HCT.

Keywords Bacterial substitution · Oral mucosa · Hematopoietic cell transplantation · Coagulase-negative *Staphylococcus* · Bacteremia

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Introduction

Allogeneic hematopoietic cell transplantation (HCT) is independently associated with increased risk of breakthrough bacteremia, which is an independent predictor of fatal outcome [1]. Coagulase-negative *Staphylococcus* (CoNS) species are the most frequently isolated bacteria from blood cultures of febrile neutropenic patients [2, 3]. These organisms are skin commensals, regarded as opportunist pathogens, particularly in association with the use of intravenous catheters [2]. On the other hand, when infections from intravenous catheters are excluded, it is

Published online: 10 June 2010

sometimes difficult to identify the focus of infection because of widespread distribution of CoNS over the body surface and their relatively large total population size [2].

Their detection may indicate not only bacteremia but also contamination of the sample [2, 4]. Determining the significance of positive blood cultures can be difficult [5–7]. Generally, the presence of clinical signs of bacteremia, identification of a possible source of infection, and/or repeated isolation of the same organism is considered suggestive of bacteremia [2, 8–10].

Allogeneic HCT often causes severe oral mucositis [11, 12], and we suspected this would be one of the major infection routes. Streptococci are common bacteria in the flora of the oral mucosa. On the other hand, in the HCT period, the bacterial flora on the oral mucosa may change because many antibiotics are used to treat the various infections that occur under neutropenic conditions. Thus, we speculated that oral mucositis may explain the CoNS infections that excluded contamination.

Previously, we reported the trajectory of oral mucositis in 127 patients undergoing conventional HCT (n=63) and reduced-intensity HCT (RIST; n=64) patients [12]. In our previous study on the trajectory of oral mucositis, we performed surveillance culture of oral buccal mucosa. Here, we performed a retrospective analysis of the bacteria on the oral mucosa after HCT in 63 of 127 patients enrolled in our previous study and for whom surveillance culture data were available.

Patients and methods

Subjects

A total of 63 patients (42 M, 21 F, 43.0±14.3 years old) who underwent allogeneic HCT at Okayama University Hospital were included in the present study. The diseases of these patients are shown in Table 1. Conventional allogeneic HCT and RIST were administered to 34 (22 M, 12 F, 34.7±11.7 years old) and 29 (20 M, 9 F, 52.8±20.3 years old) patients, respectively. The times required for engraftment (neutrophil counts >500/μL, continued for 3 days) in each group were 17.5±5.0 and 18.4±9.6 days, respectively.

Previously, we reported the trajectory of oral mucositis in 127 patients undergoing conventional HCT (n=63) and RIST (n=64). All of the subjects in the present study were included in our previous study. Thus, the trajectory of oral mucositis could be referred to from our previous report [12].

Patients had undergone bacterial examination of the buccal mucosa for oral infection control before and after HCT, and the results were analyzed retrospectively.

Informed consent for examination of oral bacteria was obtained from each subject, and the Ethical Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences approved this study (No. 263).

Table 1 Diseases of patients

Diseases	Type of HCT		Total
	Conventional	RIST	
Acute myelogenous leukemia	7	7	14
Acute lymphoblastic leukemia	7	3	10
Chronic myelogenous leukemia	1	8	9
Malignant lymphoma	12	7	19
Aplastic anemia	1		1
Myelodysplastic syndromes	6		6
Myelofibrosis		1	1
Adrenoleukodystrophy		1	1
Paroxysmal nocturnal hemoglobinuria		1	1
Metastatic renal cell carcinoma		1	1
Total	34	29	63

Conditioning regimens of HCT

Conventional allogeneic HCT

Most patients with related or unrelated donors received total body irradiation (TBI) at a dose of 12 Gy in six fractions followed by cyclophosphamide (CY) at a dose of 60 mg/kg once daily for 2 days. Alternatively, patients received a combination of busulphan (BU) (4 mg/kg/day×4 days) and CY (60 mg/kg/day×2 days). Patients with unrelated cord blood donors were treated with TBI at 12 Gy, CY (60 mg/kg/day×2 days) and cytarabine (Ara-C; 6 g/m^2/day×2 days).

RIST

Patients ineligible for the conventional myeloablative preparative regimen were treated with fludarabine (Flu)-based therapy. Patients with peripheral blood stem cell donors received Flu (25 mg/m^2/day×5 days) and CY (30 mg/kg/day×2 days). Patients with bone marrow donors received Flu (30 mg/m^2/day×6 days) and BU (4 mg/kg/day×2 days). Patients with unrelated cord blood donors received Flu (30 mg/m^2/day×6 days), CY (25 mg/kg/day×2 days), and TBI (2 Gy).

General infection control

All patients were isolated in a room equipped with a laminar airflow system and received trimethoprim-sulfamethoxazole as prophylaxis against *Pneumocystis*

carinii. Fluoroquinolone for prophylaxis against bacterial infection and fluconazole for prophylaxis against fungal infection were administered orally. Prophylaxis against herpes virus infection with acyclovir was also given. Neutropenic fever was managed according to the guidelines of Hughes et al. [13]. Briefly, empirical antibiotic therapy was administered promptly in all neutropenic patients at the onset of fever and in afebrile patients who were neutropenic but who had signs or symptoms compatible with infection. A fourth-generation cephalosporin (e.g., cefepime) or carbapenem (e.g., meropenem) was administered intravenously as empirical antibiotic therapy. G-CSF (lenograstim 5 μg/kg/day or filgrastim 300 μg/m^2) was given intravenously for 60 min starting on day 1 or 5, and was continued until the absolute neutrophil count exceeded 500/μL.

Oral management

All subjects were referred to dentists, and necessary dental treatment was completed before HCT. All subjects received instruction regarding self-management of oral hygiene; tooth brushing after every meal and before going to bed, and oral rinsing with normal saline solution every 3 h during the day was also indicated. In cases in which the patient's condition was poor, nurses, dental hygienists, and dentists performed these oral managements. No antimicrobial rinses were used.

Identification of microorganisms from the oral mucosa

Microbial identification was performed four times (day −7 to −1; day 0 to +6; day +7 to +13; day +14 to +20) for each patient (a total of 252 examinations in 63 patients).

Microbial samples were obtained about 2 h after breakfast by swabbing from the whole surface of the buccal mucosa regardless of whether mucositis was observed. Culture and identification of microorganisms were performed at the Central Clinical Laboratory of Okayama University Hospital. Microbial samples from mucosal swabs were plated onto brain heart infusion agar plates, and cultured under aerobic conditions at 37°C. Identification of colonies thus obtained was performed using rapid ID 32 STREP API®, rapid ID 32 E API®, or ID 32 GN API® identification kits (Japan bioMérieux, Tokyo, Japan) according to the manufacturer's instructions.

Analysis of relationships between antibiotic use and bacterial substitution

Antibiotic use in all patients (n=63) was examined, and the subjects were divided into two groups: one with short-term use of antibiotics (n=30, antibiotic use for 0–15 days, mean ± SD 9.9±4.7 days), and another with long-term use of antibiotics (n=33, antibiotic use for 16–28 days, mean ± SD 20.3±3.7 days). The relationships between antibiotic use

Table 2 Bacteria identified from the oral mucosa and detection frequencies before and after HCT

Bacteria	Detection number and caries frequency			
	Day−7 to −1 (n=57)	Day 0 to +6 (n=61)	Day+8 to +13 (n=60)	Day+15 to +20 (n=47)
Bacterial components of the normal flora				
α-*Streptococcus* spp.	54 (91.2%)	54 (88.5%)	34 (56.7%)*	35 (74.5%)*
γ-*Streptococcus* spp.	11 (19.3%)	11 (18.0%)	14 (23.3%)	5 (10.6%)
Neisseria spp.	35 (61.4%)	35 (57.4%)	15 (25.0%)*	8 (17.0%)*
Stomatococcus spp.	15 (26.3%)	14 (23.0%)	16 (26.7%)	14 (29.8%)
Corynebacterium spp.	0 (0.0%)	2 (3.3%)	2 (3.3%)	1 (2.1%)
Bacteria not usually found in the normal flora				
Coagulase-negative *Staphylococcus* spp.	8 (14.0%)	15 (24.6%)	33 (55.0%)*	24 (51.1%)*
Enterococcus spp.	2 (3.5%)	1 (1.6%)	3 (5.0%)	5 (10.6%)
Pseudomonas aeruginosa	1 (1.8%)	1 (1.6%)	1 (1.7%)	1 (2.1%)
Staphylococcus aureus	1 (1.8%)	2 (3.2%)	1 (1.7%)	0 (0.0%)
Bacillus spp.	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (2.1%)
Stenotrophomonas maltophilia	0 (0.0%)	0 (0.0%)	1 (1.7%)	1 (2.1%)
Haemophilus influenzae	0 (0.0%)	1 (1.6%)	0 (0.0%)	0 (0.0%)
Enterobacter cloacae	2 (3.5%)	1 (1.6%)	0 (0.0%)	0 (0.0%)

The bacteria identified on the oral mucosa are shown. Bacterial identification was performed four times (first, day −7 to −1; second, day 0 to +6; third, day +7 to +13, fourth, day +14 to +20) for each patient (total of 252 times for 63 patients). No samples were obtained in 27 of the 252 examinations because of the patients' conditions at these time points. Findings from 225 examinations are shown.

*P<0.05, χ^2 test: compared with day −7 to −1