PEDIATRIC DENTAL JOURNAL 17(1): 53–57, 2007

Bacteriological evaluation of mutans streptococci using modified mitis-salivarius-bacitracin (MSB) agar medium in primary dentition period

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Abstract Mutans streptococci considered causative agents of dental caries are indigenous to the oral cavity. Modified mitis-salivarius-bacitracin (MSB) agar medium was supplied by BML. This media has characters to grow mutans streptococci very well and to inhibit of non-mutans streptococci. However, little is known about studies using this medium. In this study, we assessed the utility of modified MSB medium and discuss the relation between mutans streptococci and dental caries in primary dentition period. Modified MSB medium was found to be a suitable medium to isolate and quantify mutans streptococci since it could permit selective growth of mutans streptococci. Moreover, this medium inhibited to non-mutans streptococci (*S. anginosus* and *S. intermedius*), completely. The detection rate of *Streptococcus mutans* and *Streptococcus sobrinus* increased in proportion to the severity of dental caries in the nursery children. From these results, modified MSB agar medium is useful in the judgment to detect the mutans streptococci.

Key words
Dental caries,
Modified mitis-salivarius-bacitracin (MSB) agar medium,
Polymerase chain reaction (PCR),
Streptococcus mutans,
Streptococcus sobrinus

Introduction

Streptococcus mutans and *Streptococcus sobrinus* (mutans streptococci) have been implicated in the etiology of human dental caries[1–3]. Epidemiological studies have revealed a strong correlation between the incidence and progress of dental caries and amount of *S. mutans* in the oral cavity[4,5]. A possibility of transmission of the oral bacterial flora from mothers to children has also been indicated[6,7]. Therefore, investigation of the infectious conditions of *S. mutans* and/or *S. sobrinus* seems to be important to prevent caries incidence.

Several selective agar media for mutans streptococci such as mitis-salivarius (MS) agar[8] and mitis-salivarius-bacitracin (MSB) agar[9] (MS agar containing 200 U of bacitracin and 15% sucrose per liter) have been developed. However, non-mutans streptococci were found to form the colonies as well as mutans streptococci in these selective media[10]. Then modified MSB medium containing three kinds of antibiotics (2 mg of gramicidin D, 10 mg of colistin, and 10 mg of nalidixic acid per liter) in addition to bacitracin was supplied by BML (Tokyo, Japan)[11]. In this medium mutans streptococci can grow well, but only poor growth of non-mutans streptococci is permitted. Furthermore, *S. mutans* and *S. sobrinus* are distinguishable (by naked eyes) based on the different properties of colonies on modified MSB agar. The aim of this study is to assess the utility of this medium and discuss the relation between mutans streptococci and dental caries in primary dentition period.

Received on September 30, 2006
Accepted on January 26, 2007

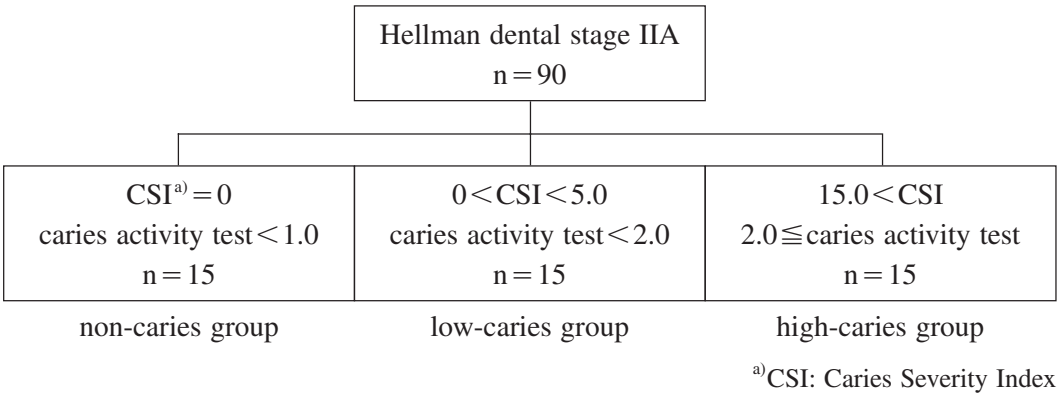


Fig. 1 Criteria in classified three caries groups

Table 1 Nucleotide sequences of the primers used in this study

		sequence (5′-3′)	amplified size (bp)
S. mutans	Sm-BF	CACTATCGGCGGTTACGAAT	197
	Sm-BR	CAGCAATTTGGAGCAAGTCA	
S. sobrinus	Ss-16sF	ACCGCATAAGAGGAGTTAACT	323
	Ss-16sR	GGTACCGTCACTGTGTAAGC	

Materials and Methods

Growth of streptococci in the selective media

To assess the growth of MS (*S. mutans* Ingbritt and MT8148, and *S. sobrinus* 6715 and GTC274) in MSB and modified MSB agar, these bacteria were subcultured separately in brain heart infusion (BHI, Becton Dickinson Co., MD., USA) broth in a glove box filled with mixture of gases ($N_2 + CO_2 + H_2$; 85 : 10 : 5). The same volumes (0.1 m*l*) of each culture which was diluted adequately in the sterile saline was inoculated onto MSB and modified MSB agar plates followed by incubation at 37°C anaerobically for 72 hours. Similarly, the growth of non-mutans streptococci (*Streptococcus anginosus* ATCC 33397 and *Streptococcus intermedius* ATCC 27335) was assessed. The colonies of each agar plate (MS, MSB, and modified MSB) were counted after the incubation and the numbers of bacteria were indicated as CFU/m*l*.

Bacteriological evaluation of dental caries in primary dentition period

Oral examination of 90 nursery children was done who corresponded to Hellman dental stage IIA in Nagano prefecture. In the process of these operations, the clinician undertook the inspection of the oral cavities in the supine position using dental mirrors in accordance with the general criteria of dental caries. And then, we carried out caries activity test using Mucount® and Cariostat®, calculated caries severity index (CSI) in all subjects. In accordance with these methods, they were classified into three groups (non-caries group, low-caries group, and high-caries group), 15 children were chosen randomly from each group (Fig. 1). Saliva samples ware collected using sterile cotton-swab. Collection of dental plaque materials were performed by the following methods; dental plaque materials were recovered in 0.5 m*l* of gargling liquid saline after one minute of tooth brushing of each subject by the clinician. Each sample was incubated in modified MSB agar as described anaerobically.

Colony-direct PCR was carried out to confirm that the colonies formed on modified MSB agar from saliva and dental plaque were really *S. mutans* or *S. sobrinus*. The oligonucleotide primers are listed in Table 1. The primers of PCR test for *S. mutans* (Sm-BF and Sm-BR) were the internal sequence of *gtfB* of *S. mutans*. The corresponding

Table 2-1 Comparison of growth of mutans streptococci in the two agar medium

Ave.: CFU/m*l*

bacteria \ media		MSB agar		modified MSB agar
S. mutans	Ingbritt	$5.1\times10^{8}\ \pm2.8\times10^{8}$	*	$5.3\times10^{9}\ \pm1.9\times10^{9}$
	MT8148	$5.7\times10^{8}\ \pm5.2\times10^{7}$	**	$1.2\times10^{10}\pm1.5\times10^{9}$
S. sobrinus	6715	$1.4\times10^{8}\ \pm6.0\times10^{7}$	ns	$2.7\times10^{8}\ \pm1.4\times10^{8}$
	GTC274	$3.7\times10^{7}\ \pm3.1\times10^{7}$	ns	$4.6\times10^{7}\ \pm3.7\times10^{7}$

mean ± S.D.
ns: not significant, *: $P<0.05$, **: $P<0.01$

Table 2-2 Comparison of growth of non-mutans streptococci in the three agar medium

Ave.: CFU/m*l*

bacteria \ media		MS agar		MSB agar	modified MSB agar
S. anginosus	ATCC 33397	$6.6\times10^{8}\ \pm2.6\times10^{8}$	ns	$3.0\times10^{8}\ \pm8.6\times10^{7}$	trace
S. intermedius	ATCC 27335	$4.2\times10^{10}\pm2.7\times10^{9}$	**	$8.8\times10^{5}\ \pm1.8\times10^{5}$	trace

mean ± S.D.
ns: not significant, **: $P<0.01$

primers for *S. sobrinus* (Ss-16sF and Ss-16sR) were derived from 16S rRNA of *S. sobrinus*. Colonies taken from the agar plates were applied to Takara PCR Thermal Cycler Dice (Takara Bio, Kyoto, Japan) and subjected to the treatments including the following parameters; a preincubation step at 95°C for 5 min, followed by 35 cycles denaturating step at 95°C for 30 sec, primer annealing step at 60°C for 1 min, and extension step at 72°C for 1 min, and an extra-extension step at 72°C. PCR products were examined on agarose gel (2%) electrophoresis for the identification. The presence or absence of bands was noted.

Statistical analysis was performed using *t*-test (Tables 2-1 and 2-2) and χ^2 test (Fig. 2).

This work was undertaken with the approval of the Ethics Committee of Matsumoto Dental University. The consent of the subjects and guardians was obtained after the sufficient explanations of the work.

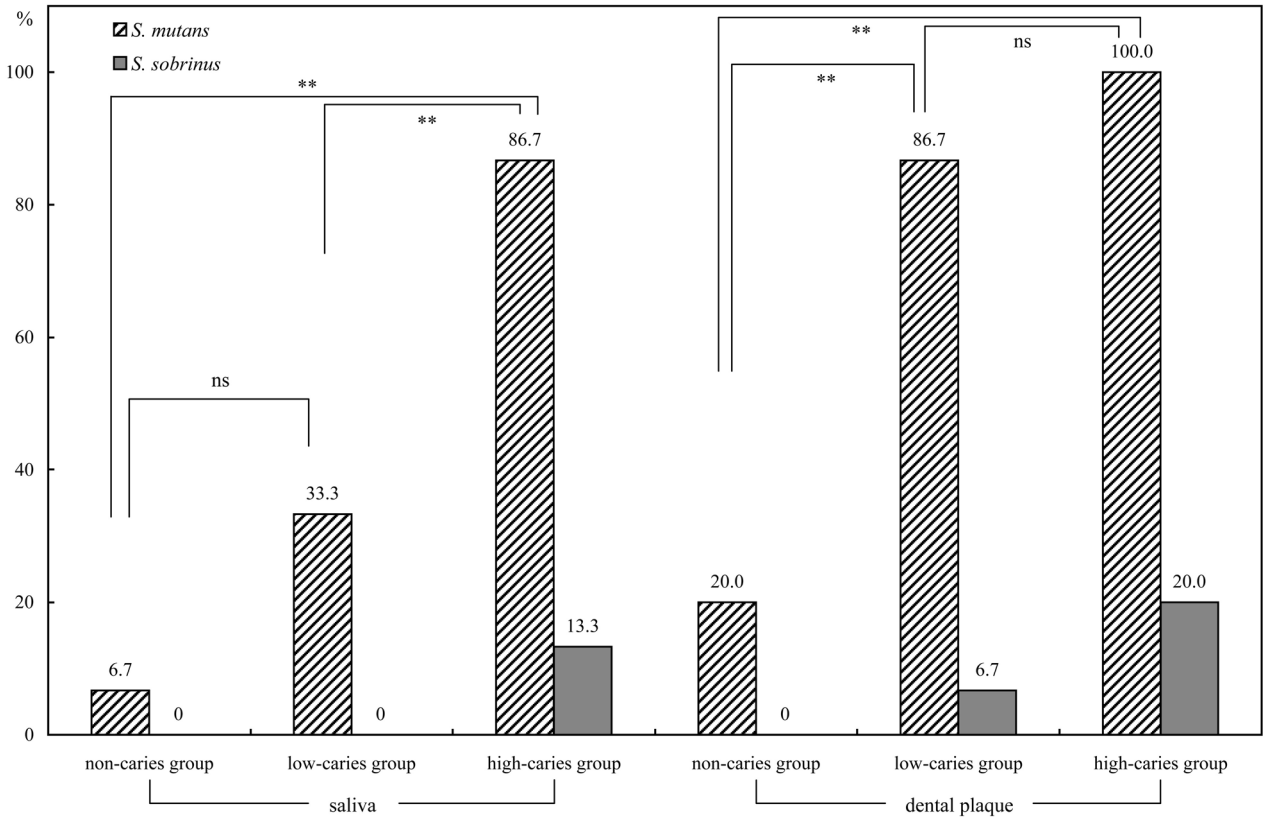
Results

Growth of oral streptococci in MS, MSB and modified MSB agar plates

Comparison of growth of mutans streptococci and non-mutans streptococci is summarized in Tables 2-1 and 2-2. The colony forming units of *S. mutans* and *S. sobrinus* in modified MSB agar were larger than those in MSB agar. *S. anginosus* appeared to be resistant to bacitracin, while *S. intermedius* was fairly sensitive to it. However, inhibition of growth of these species by modified MSB medium was remarkable. This medium supported only negligible growth of the non-mutans streptococci. Contrarily, no inhibition of mutans streptococci was observed in modified MSB medium.

Relation between detection rates of mutans streptococci and dental caries in primary dentition period

Detection of mutans streptococci from 15 subjects of each caries group was examined (Fig. 2). The detection rate of *S. mutans* from saliva was 6.7% in non-caries group, 33.3% in low-caries group, and 86.7% in high-caries group, respectively. It is notable that of *S. sobrinus* was detected only from high-caries group with significant rate (13.3%). The detection rate of *S. mutans* from dental plaque was 20.0% in non-caries group, 86.7% in low-caries group, and 100.0% in high-caries group, respectively. Furthermore, *S. sobrinus* was only found in low-caries group (6.7%) and high-caries group (20.0%).



ns: not significant, **: $P<0.01$

Fig. 2 Percentile detection rate of *S. mutans* and *S. sobrinus* from the three caries groups

Discussion

Quantitative analyses of mutans streptococci in the oral cavity have been considered to be worthy for the epidemiological studies of dental caries[12,13]. MS, MSB media, and Cariostat® are frequently used for the detection and confirmation of these microorganisms[12,14]. However, significant amounts of non-mutans streptococci were found to grow in MS and MSB media[10] as we described. Modified MSB medium was developed with special regard to this awkward problem in the caries related bacteriological examinations[15,16]. We observed that mutans streptococci showed good growth in modified MSB agar medium but non-mutans streptococci did not (Table 2-2). Modified MSB medium, consequently, was employed to detect mutans streptococci from saliva and dental plaque in the future.

Since PCR is a rapid and suitable method for the identification of bacterial species, it has been employed to identify the oral streptococci[14,17–19]. The colonies grown in modified MSB agar medium have been almost rightly identified to distinguish by characters of the colonies formed as a result of colony-direct PCR.

Among the detection rate of *S. mutans* in the three caries groups the most numerous was the high-caries group, the second was low-caries group, and the third was non-caries group. However, *S. sobrinus* was not found in non-caries group (Fig. 2), indicating the contribution of *S. sobrinus*, whether essential a not, to progress of dental caries. Hirose *et al.*[13,20] reported it is correlation between CSI and numbers of *S. mutans*. We examined it from dental plaque and saliva samples in the materials. It was confirmed plaque samples were more correlative than saliva (data not shown). Regarding bacterial samples collected from the oral cavity, it has been reported that more stable results can be obtained from saliva than dental plaque[19,21]. On the other hand, Fukushima *et al.* reported that the numbers of mutans streptococci obtained after brushing dental plaque did not exert the fluctuation of mutans streptococci from experiment to experiment, and

it was recommended to use the dental plaque for quantitative bacterial investigations of caries[11,22]. In conclusion, modified MSB agar medium may be useful for the epidemiological studies of dental caries.

Acknowledgments

We are greatly gratitude to Prof. T. Maeda, Department of Pediatric Dentistry, Prof. K. Fukushima, Department of Oral Bacteriology, Nihon University School of Dentistry at Matsudo, and Prof. S. Fujimura, Department of Oral Microbiology, Matsumoto Dental University for suggestion and advice.

This study was supported in part by a Grant-in-Aid Scientific Research (No. 16791303 to Tamami Saito) and a Scientific Research Special Grant from Matsumoto Dental University (to Tamami Saito).

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