Identification of Non-*Streptococcus mutans* Bacteria from Predente Infant Saliva Grown on Mitis-Salivarius-Bacitracin Agar

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Objective: *Although mitis-salivarius-bacitracin (MSB) agar is a commonly used selective medium for detecting Streptococcus mutans in clinical studies, non-S. mutans microorganisms are cultivatable on MSB agar. Since few studies have identified non-S. mutans bacteria grown on MSB, this study aimed to identify and differentiate MSB-grown non-S. mutans bacteria from predente infants' oral cavity.* ***Study design:*** *The saliva from 51 predente infants were plated on MSB agars. Bacteria colonies were characterized based on their morphology under direct visualization and light microscopic observation. Colony PCR targeting S. mutans htrA locus and 16S rRNA DNA sequencing were used for further bacteria identification.* ***Results:*** *Overall, 80% of the predente infants had oral bacteria grown on the MSB agar. Nine bacteria were identified, including S. mutans, Staphylococcus epidermidis, Klebsiella quasi-pneumoniae, Klebsiella pneumoniae, Enterobacter kobei, Enterococcus faecalis, Staphylococcus hominis, Streptococcus anginosus and Phytobacter. The most frequently detected bacteria were S. epidermidis (41.5%), followed by E. kobei (24.4%), K. pneumoniae (17.1%) and S. mutans (9.8%.)* ***Conclusions:*** *Multiple non-S. mutans bacteria from infants' oral cavity could grow on MSB agar. Caution should be exercised in counting the colony forming units of S. mutans from oral samples on MSB agar to avoid overestimation by assuming that all colonies on the MSB agar are S. mutans. Using the colony morphological guide we summarized, these non-S. mutans bacteria could be distinguished from S. mutans. Our study provides a key reference to pediatric cariology clinical-epidemiological studies that commonly use MSB to identify/quantify S. mutans in infants and young children.*

Keywords: *Oral bacteria, Mitis-salivarius-bacitracin agar, Predente infants, Saliva, Streptococcus mutans, Colony morphology.*

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INTRODUCTION

Dental caries is a chronic infectious disease, initiated from the virulent dental biofilms/plaque formed on tooth surfaces [1]. Within the dental biofilms/plaque, oral cariogenic bacteria metabolize dietary carbohydrate, produce acid and initiate demineralization of tooth enamel, and further leads to dental caries [2]. *Streptococcus mutans* has been considered as a well-known culprit for dental caries [3] including early childhood caries (ECC) [4-10], due to its acidogencity, aciduricity and capability of synthesizing dental plaque extracellular matrix [4,11-14].

Various culture-independent methods have been used to identify and quantify oral *S. mutans* carriage, including biochemical tests [15], DNA probes [16], polymerase chain reaction-restriction (PCR) [17] and 16S rDNA sequencing comparison methods[18]. Although culture-independent methods have been widely used to identify the presence and measure relative abundance of *S. mutans* in oral samples, the critical quantitative caries risk indicator remains to be that individual with more than 10^5 colony forming unit (CFU) /ml of salivary *S. mutans* are considered as high risk for caries [19-26]. Therefore, isolation, identification and quantification of the *S. mutans* from oral samples (saliva, plaque and swab) using culture-dependent methods

still present as essential components for clinical and epidemiological studies, in addition to other culture independent assays [27-30].

Mitis-salivarius-bacitracin (MSB) agar is a commonly used selective medium for isolating and quantifying *S. mutans* in many cariology clinical and epidemiological studies [27,31-34]. However, previous reports have discussed the non-specificity of isolating *S. mutans* using MSB agar [35-37]. For instance, *S. sobrinus* could also be isolated from the oral cavity of young children using MSB [36]. Nevertheless, few studies have described and identified other non-*S. mutans* species isolated from MSB [38,39]. Moreover, our prospective birth-cohort study that examined the colonization of *S. mutans* in infants have observed multiple bacteria that grew on the MSB, in addition to *S. mutans*. Therefore, the aim of this study was to characterize, identify and differentiate these non-*S. mutans* bacteria isolated from predente infants saliva samples using MSB agar, which could serve as a key reference to cariology clinical-epidemiological studies that commonly use MSB to identify and quantify *S. mutans* in infants and young children.

MATERIALS AND METHOD

Study Subject Enrolment and Clinical Sample Collection

This study was approved by the University of Rochester Institutional Review Board (#67191). Infants met the following conditions were excluded: born pre-term (<37 weeks of gestation at birth), with low birth weight (<2500), with Orofacial deformity (cleft lip, cleft palate, oral-pharyngeal mass), with Down syndrome, immediately admitted into the Neonatal Intensive Care Unit (NICU) after birth and stayed in NICU less than two weeks, or received antifungal oral and/or systematic treatment before the study visit.

The saliva samples from 51 healthy infants (0-6 months) were collected using SalivaBio Infant's Swab (SIS, Salimetrics, Inc. Carlsbad CA, USA). A trained dentist securely held one end of the SIS swab and placed the other end under the infant's tongue (when possible) or at the corners of the mouth. Infants were allowed to chew on the swab. Saliva were collected in intervals by resting the swab inside the mouth for 20 sec, and then re-introducing into the infants' mouth for another 20 sec. Saliva collection continued until the lower third of the swab is saturated (some participants required up to 90 seconds of total collection time). After the saliva collection, the SIS was stored in a sterilized tube and kept on ice and transferred to the laboratory located at the University of Rochester Center for Oral Biology within two hours.

MSB Agar Preparation and Infant Saliva Sample Processing

To prepare the MSB agar, 54 g mitis salivarius agar and 90 g sucrose were added in 600 ml distilled H_2O. The mixture was autoclaved. After the temperature cooled down to 50 °C, 700 µl of 1% tellurite solution and 700 µl of 3 mg/ml bacitracin were added. Poured agar plates were left at room temperature overnight. Infant saliva samples were centrifuged for 15 minutes at 1500 g to obtain infant saliva. In general, 400-1000 µl of saliva was harvested from the centrifuging process. 50 µl of saliva suspensions with no dilution, and with 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated onto MSB plates in duplicates and incubated in 5% CO_2 at 37 °C for 72 hours.

Bacteria Identification

Three methods were used to identify bacteria isolated from MSB agar, including colony morphology differentiation [40], colony PCR with *S. mutans* specific primers and 16s rRNA gene sequencing. *S. mutans* UA159 (ATCC® 700610™) and *S. sobrinus* (ATCC® 33478™) were used as controls.

1. Colony Morphology Differentiation

After 72-hour incubation, bacteria colonies isolated from the MSB plates were observed with naked eyes and under the light microscope (40×, Olympus, Japan). Direct photos and light microscope images were obtained.

2. Colony PCR Targeting S. mutans

Three to five colonies were picked from MSB agar plates and added into the PCR strip tube with a mixture of GoTaq® colorless master mix (Promega, USA) and primer pair Sm479F/R (Sm479F: 5'-TCGCGAAAAAGATAAACAAACA-3' and Sm479R: 5'-GCCCCTTCACAGTTGGTTAG-3') [41]. This primer set has been used previously to identify *S. mutans by* targeting the segment comprises a portion of the htrA locus and a part of an intergenic locus of the *S. mutans* genome [41]. PCR were performed on a Thermocycler (Thermo Fisher Scientific, USA). The PCR reaction conditions were: 1) 5 min at 95°C, 2) 35 cycles of: 15 sec at 95°C, 30 sec at an annealing temperature 55°C and 1 min at 72°C, 3) followed by 10 min at 72°C. The PCR amplicon product (479 bp) was imaged using an Invitrogen™ E-Gel™ Power Snap Electrophoresis Device and 2% EX gel (Thermo Fisher Scientific, USA).

3. 16S rRNA Gene Sequencing

Bacteria genomic DNA was extracted from the overnight bacteria liquid culture medium using MasterPure DNA Extraction Kit (Epicentre, USA). Extracted DNA was evaluated for quality and quantity using the NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). Total DNA was used as a template for PCR amplification of 1.4 kbp fragment of the 16S ribosomal RNA gene. Standard 16S ribosomal RNA PCR primers (27F and 1492R) were used. Water was used as a negative control. All PCR fragments were analyzed for quantity and quality by agarose gel electrophoresis. All PCR fragments were sequenced using the 27F and 1492R primers. All sequencing was performed with the BigDye Terminator Cycle Sequencing Kit, V 3.1, and analyzed on an ABI 3730XL Genetic Analyzer. All sequence reactions were then evaluated and aligned. Consensus sequences were generated for each fragment and compared with the existing species database (NCBI) by the BLAST search algorithm.

RESULTS

Non-S. mutans bacteria strains grown on MSB agar

Overall, 80% (41/51) of the predente infants had bacteria grown on the MSB agar. Nine bacteria strains including *S. mutans* and another eight non-*S. mutans* were isolated from predente infants' saliva samples using MSB agar. These bacteria were further identified using 16S rRNA gene sequencing, including *S. mutans, Staphylococcus epidermidis*, *Klebsiella quasi-pneumoniae*, *Klebsiella pneumoniae*, *Enterobacter kobei*, *Enterococcus faecalis*, *Staphylococcus hominis*, *Streptococcus anginosus* and

Phytobacter. The colony morphology from direct visualization and light microscopic observation are summarized in Figure 1 and Table 1. Bacterial characteristics, results from colony PCR that targeted *S. mutans htrA* locus and 16S rRNA gene sequencing identification and clinical relevance were also presented in Table 1. Regarding the colony morphology, *S. mutans* (A-9 and B-9) and *S. sobrinus* (A-10 and B-10) shared similar colony characteristics; both of them had unique morphology of frosted glass colonies which rooted into the agar. The colonies of *S. sobrinus* were surrounded by more distinct polysaccharide.

Despite the similarity between *S. mutans* and *S. sobrinus* colonies, all other non-*S. mutans* bacteria that grew on the MSB agar were distinguishable based on their colony morphology using our summarized colony morphological guide in Figure 1 and Table 1. The colony size (diameter) of the MSB isolated oral bacteria ranged from 0.5 mm to 4 mm. *S. epidermidis, E. kobei* and *S. hominis* tended to form larger colonies, with an average size 2-4 mm. The colonies of *S. mutans*, *S. sobrinus, S. anginosus* and *Phytobacter* were on the smaller scale, with an average size of 0.5-1 mm. Majority of the colonies were dark black (except for *K. pneumonia* and *E. kobei*). Several bacteria colonies were accompanied by distinct smell, e.g. strong sour fermentation smell (*E. kobei*) and sweet smell (*K. pneumonia*). Four bacteria did not have distinct smell (*S. hominis*, *S. anginosus, S. mutans* and *S. sobrinus*).

The clinical relevance of these MSB-isolated bacteria was summarized in Table 1. *S. mutans* and *S. sobrinus* were considered as cariogenic strains. Several other bacteria were considered as pathogens for nosocomial infection. For example, *K. pneumonia* was closely associated with pneumonias, urinary tract infections, bacteremias, and liver abscesses [42], and *E. faecalis* could cause endocarditis, bacteremia, urinary tract infections, meningitis, and other fatal forms of systemic and local infection [43].

Bacteria Detection Frequency and Quantity

The bacteria detection frequency and quantity were shown in Table 2. Eighty percent (41/51) of the infants' saliva had bacteria that grew on MSB agar. Among the isolated bacteria grown on MSB, *S. epidermidis* was the most frequently detected bacteria (41.5%), followed by *E. kobei* (24.4%), *K. pneumoniae* (17.1%) and *S. mutans* (9.8%). *E. kobei* was the most abundant bacteria grown on MSB (CFU/ml $1.7\pm4.4\times10^7$), whereas *S. hominis* was the least abundant bacteria grown on MSB.

DISCUSSION

Culture-dependent methods that use MSB medium to isolate, cultivate, identify and quantify *S. mutans* are commonly used in cariology clinical and epidemiology studies. In contrast to the popularity of using MSB agar in these studies, limited selectivity of using MSB to isolated/identify *S. mutans* from predente infants' saliva had not been reported. Moreover, limited study have characterized and identified non-*S. mutans* microorganisms isolated from MSB. To fill the gap, our study detailed the characteristics and differentiation of MSB-isolated non-*S. mutans* bacteria, which could be used as important references for cariology clinical and epidemiological studies. Our study identified eight non-S. *mutans* bacteria isolated from infants' saliva using MSB. Several bacteria shared similar appearance with *S. mutans* on the MSB. For instance, *S. sobrinus*, *S. anginosus* and *Phytobacter*. Without proper observation and differentiation, these colonies could be falsely recognized as *S. mutans,* which might lead to inflated positive *S. mutans* detection in clinical studies.

The mutans streptococci constitute a group of oral cariogenic species, with *S. mutans* and *S. sobrinus* being the most frequently detected strains from the oral cavity of individuals at high risk for dental caries [44-46]. *S. mutans* has been detected from ~80% to 90% of adults' and ~50% to 100% of preschool children's oral cavity [27], but much less frequent (~10%) in the oral cavity of infants [47,48]. The oral colonization by *S. mutans* is considered to take place around 26 months [49]. Li *et al* showed the *S. mutans* detection rate at 70-80% from old children aged three and five [50]. Our study showed that *S. mutans* presented in predente infants' oral cavity, with a detection rate of 9.8%. Interestingly, *S. sobrinus* was not detected from any of the 51 predente infants.

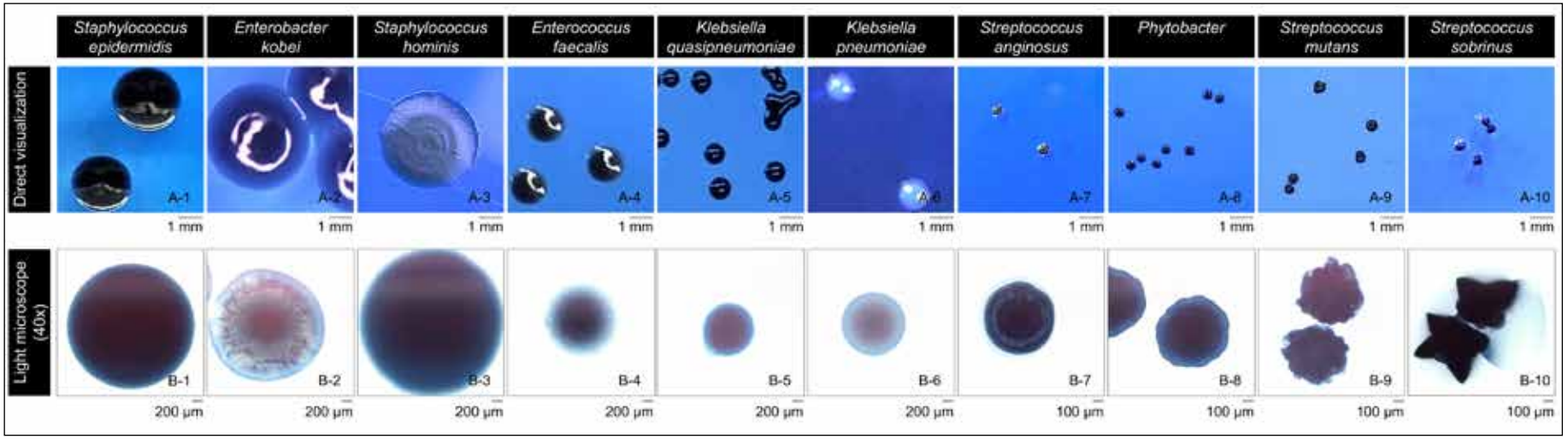
The oral cavity is considered as sterile at birth, and quickly colonized by multiple microorganisms through infants' breathing, feeding and contacting with family, medical personals and caregiver [51]. Understanding the development and diversity of human oral microorganisms in early infancy is important to understanding the factors that relate to oral health and disease from a microbiological standpoint. In this study, we showed that *S. epidermidis*, *E. kobei* and *K. pneumoniae* were colonized in predente infants' mouth. As reported previously, *E. faecalis*, *E. coli*, *Enterobacter cloacae*, *K. pneumoniae*, *S. epidermidis* and *Staphylococcus haemolyticus* were found as predominant species in infants' intestinal microflora [52]. These three bacteria are commonly recognized as nosocomial pathogens, [42,53,54]. *S. epidermidis* is found ubiquitously on healthy human skin and mucosal surfaces [55], readily colonizing newborns [56] and remaining part of the normal microflora throughout life [57], and it rarely causes infections in healthy tissue [58]. *E. kobei* is a new species of the family *Enterobacteriaceae* resembling *Enterobacter cloacae* [59], which has been reported as an important opportunistic and multi-resistant bacterial pathogen for humans in hospital wards [60]. *K. pneumoniae's* virulence factors are closely associated with pneumonia infections [42]. It is speculated that most of the eight non-*S. mutans* bacteria isolated from MSB might be transient species due to the particularities of neonatal innate immunity.

In summary, eight non-*S. mutans* bacteria strains from 51 predente infants' saliva grew on the MSB agar and were identified using 16S rRNA sequencing. Caution should be exercised in counting the colony forming units of *S. mutans* from oral samples on MSB agar to avoid overestimation by assuming that all colonies on the MSB agar are *S. mutans*. Overall, considering the non-*S. mutans* bacteria colonies were distinguishable from *S. mutans* based on their colony morphological characteristics, the lower cost and better affordability when compared to 16S rRNA sequencing, our study results suggest that MSB agar could still be used for initial identification/quantification of *S. mutans* in clinical and epidemiology study setting, followed by further validation using colony PCR that targets the *S. mutans* specific locus.

Table 1 Colony morphology and description of bacteria from predente infant oral cavity grown on MSB agar

Bacteria	*Staphylococcus epidermidis*	*Enterobacter kobei*	*Staphylococcus hominis*	*Enterococcus faecalis*	*Klebsiella quasi-pneumoniae*	*Klebsiella pneumoniae*	*Streptococcus anginosus*	*Phytobacter sp.*	*Streptococcus mutans*	*Streptococcus sobrinus*
Colony size (diameter)	2~4 mm	2~4 mm	2~3 mm	1~1.5 mm	~1 mm	~1 mm	~0.5 mm	~0.5 mm	0.5~1 mm	0.5~1 mm
Colony morphology	Dark black, perfect round, regular margin, flat, shiny	Bright blue, iris, bull-eye, mucoid, easy to form connected colonies	Black blue, round, flat, matt-finish, wrinkles on the colony edge	Dark black or gray, round, small	Dark black, shiny, round, tall, easy to form chains	Light blue or gray, small dome	Dark black, small dome, irregular round, textured surface	Dark black, shiny, irregular margin	Frosted glass, irregular margin, black blue, rooted into the agar, star-shaped	Frosted glass, irregular margin, black blue, rooted into the agar, surrounded by polysaccharide
Colony smell	Slightly fermented	Fermented, sour	No distinct smell	Foul smell	Slightly fermented	Fermented, slightly sweet	No distinct smell	Slightly sour	No distinct smell	No distinct smell
S. mutans Colony PCR	-	-	-	-	-	-	-	-	+	-
Bacteria characteristics	Gram-positive Facultative anaerobic Typically found in the skin flora One of the most commonly etiological agent of nosocomial infections [53]	Gram-negative Oxidase-negative Fermentative rods Facultative anaerobic *Enterobacter cloacae* complex (*E. Kobei* included) is one of the important nosocomial pathogens, responsible for 65%–75% of all infections [54]	Gram-positive Limited facultative anaerobic Potentially opportunistic pathogens, could cause bloodstream infections, endocarditis, peritonitis, osteomyelitis, bone and joint infections [61] Commonly recoverable from the blood of hospitalized patients [62]	Gram-positive Facultative anaerobic One of the most commonly etiological agent of nosocomial infection Could cause endocarditis, bacteremia, urinary tract infections, meningitis, and other fatal forms of systemic and local infection [43] Frequently found in re-infected, root canal-treated teeth [63]	Gram-negative Oxidase-negative Rod-shaped bacteria with a prominent polysaccharide-based capsule Similar to *K. Pneumonia* [64]	Gram-negative Facultative anaerobic Rod-shaped Commonly found in the normal flora of the mouth, skin and intestines Could cause a wide range of infections, including pneumonias, urinary tract infections, bacteremias, and liver abscesses [42]	Gram-positive Facultative anaerobic Commonly found in the oral cavity gastrointestinal and urogenital tract. Potential pathogenic role in cystic fibrosis.[65]	Gram-negative Facultative anaerobic	Gram-positive Facultative anaerobic Commonly found in the oral cavity Cariogenic bacteria [21,44,51,66]	Gram-positive Anaerobic Closely related species of *S. mutans* Cariogenic bacteria[44,67]

Figure 1. Colony morphology of bacteria grown on MSB agar



*Nine bacteria strains (A-1 to A-9 and B-1 to B-9) from predente infants' saliva sample grew on MSB agar. A-10 and B-10 were *Streptococcus sobrinus* (ATCC® 33478™) wild strain.

Table 2 Detection frequency and quantity of bacteria strains isolated by MSB agar from predente infants (0-6 months old) (n=51)

Bacteria	*Staphylococcus epidermidis*	*Enterobacter kobei*	*Staphylococcus hominis*	*Enterococcus faecalis*	*Klebsiella quasi-pneumoniae*	*Klebsiella pneumoniae*	*Streptococcus anginosus*	*Phytobacter sp.*	*Streptococcus mutans*	*Streptococcus sobrinus*
Detection frequency	41.5%	24.4%	2.4%	12.2%	2.4%	17.1%	2.4%	2.4%	9.8%	0
Detection quantity (CFU/ml, Mean ± SD)	$6.7 \pm 12.1 \times 10^2$	$1.7 \pm 4.4 \times 10^7$	1.5×10^1	$6.7 \pm 8.8 \times 10^3$	9.7×10^1	$3.9 \pm 5.5 \times 10^2$	4.5×10^1	1.1×10^5	$1.7 \pm 3.4 \times 10^5$	0

CONCLUSIONS

Our study indicated that a variety of non-*S. mutans* bacteria from predente infant's saliva could grow on MSB agar, including *S. epidermidis*, *K. quasi-pneumoniae*, *K. pneumoniae*, *E. kobei*, *E. faecalis*, *S. hominis*, *S. anginosus*, *Phytobacter*. Using the colony morphological guide we summarized, these bacteria could be distinguished from *S. mutans*, which provides an important key reference to cariology clinical-epidemiological studies that commonly use MSB selective medium to identify and quantify *S. mutans* in infants and young children.

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Tables and Figures