

Caries-related Microbiological Screening in Children under Three Years of Age

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국문초록

3세 이하의 어린이에서 구강 내 미생물과 타액완충능이 치아우식증과의 관련성을 알아보았다. 87명의 어린이를 실험 대상으로 하여 치태와 자극성타액을 각각 면봉과 면구를 이용하여 채취하였다. 0.94%의 lactic acid를 타액 표본에 첨가하기 전과 후의 pH를 각각 측정하였고 타액표본을 순차적으로 희석하여 선택배지와 비선택배지에 접종하였다. Mutans streptococci (MS), lactobacilli (LB), total viable count (TVC)의 집락수와 타액의 pH와 완충능을 어린이의 치아우식과 비교하여 다음과 같은 결과를 얻었다.

1. 자극성타액과 치태 모두에서 MS 와 LB 가 치아우식증과 높은 통계학적 유의성을 보였다.
2. 치아우식의 발생을 예측할 수 있는 미생물의 집락수는 다음과 같았다.
 - 1) 자극성타액 1 ml 당 MS 집락수가 10^5 이상
 - 2) 치태 1 ml 당 MS 집락수가 2×10^5 이상
 - 3) 자극성타액 1 ml 당 LB 집락수가 10^3 이상
 - 4) 치태 1 ml 당 LB 집락수가 10^3 이상
3. 타액의 pH와 완충능은 유아기우식증과 관련이 없었다.
4. MS 검사는 LB 검사 보다 높은 예측치 (predictive value)와 교차비 (odds ratio)를 보였다.
5. MS 집락수는 어린이에 있어서 치아우식 발생을 예측할 수 있는 가장 믿을만한 미생물학적 검사로 일반적인 세균배양법을 이용하거나 상품화된 검사기구를 이용하여 쉽게 임상에 적용할 수 있다.

주요어 : 타액완충능, 치태, 자극성타액, mutans streptococci, lactobacilli

I. Introduction

Dental caries is the most widespread chronic disease as well as the most common unmet health care need of childhood¹⁾. Treatment of early childhood caries (ECC) is often requiring extensive restorative treatment and extraction of teeth at an early age,

and general anesthesia or deep sedation may be required because such young children lack the ability to cope with the procedures²⁾. Children with ECC, additionally, are at higher risk for development of new carious lesions at a later age³⁾.

The pathogenicity of cariogenic bacteria in ECC has been clearly established. Mutans streptococci (MS) is transmitted from the child's caregiver (usually the mother) to the child during infancy⁴⁾. The transmission of MS from the mother appears to be an important factor in ECC. Mothers with high oral levels of MS transmit more MS to their infants and at an earlier time (relative to the infant's age), than mothers

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whose salivary MS fall down below $10^5/\text{ml}$ ^{5,6)}. Dental caries in the child by the age of 4 inversely correlates with the time of infant colonization by MS and directly correlates with MS infection level of the mother^{5,6,7)}. Early colonization patterns therefore should be closely monitored in children shortly after teeth erupt, and interventions implemented if necessary in order to prevent ECC prior to lesion formation.

Microbial screening tests have been utilized extensively to identify older children and adults, and to a lesser extent in infants and toddlers, who are at risk for development of dental caries⁷⁻¹⁵⁾. In older individuals, these tests usually involve quantitation of the cariogenic bacteria, i.e., mutans streptococci and lactobacilli (LB) in stimulated saliva^{8,9)}, whereas in young children, tongue specimens collected with wooden or plastic tongue blades or the backs of mouth mirrors which are subsequently inoculated on selective agar media are frequently employed^{11,12,16-18)}. Some investigators have sampled other oral sites, such as plaque or the buccal mucosa, or utilized conventional dilution and plating methods^{10,14,19-22)}.

Although most evaluations of microbial screening have been conducted in individuals with mixed or permanent dentitions, the same microbiological/dental caries relations are believed to be present in children with incomplete or complete primary dentitions^{23,24)}.

The general purpose of the present investigation was to examine association of caries status with colony-forming units (CFU) of MS and LB or salivary variables including salivary pH and salivary buffer capacity in children under three years of age. Concurrently, the level of carious activity was measured and compared to the level of MS and LB, to determine the quantitative values that are associated with caries activity. These methods may be useful clinically to identify young children at risk for caries development.

II. Materials and methods

1. Patient Selection and Examination

Eighty-nine children, 36 months of age or younger were sequentially selected from the patients or younger siblings of the patients attending the

Pediatric Dentistry clinic at the University of Maryland Dental School, or children who attended the Well Baby Clinic at the University of Maryland Pediatric Ambulatory Center in Baltimore inner-city. Main criteria for the selection were presence of at least 2 erupted teeth and patient age. Patients who received antibiotics within 14 days were excluded as well as patients who received a professional fluoride treatment within 48 hours of the microbial specimen collection. All the patients were deemed medically healthy through assessment of the patient's medical history. The protocol and consent form were approved by the Institutional Review Board for the University of Maryland at Baltimore.

Prior to data collection, calibrations were performed by means of blinded repeated children examinations by the examiner and a faculty member with experience in oral health survey calibrations and clinical examination of ECC research, under similar dental exam environment conditions. Kappa scores for diagnosis agreement of dental cavitation, presence of opacities and white-spot lesions (WSL) were calculated for the total dentition. There was an inter-examiner agreement (kappa statistic) of 93% between the two examiners for the presence of dental cavitation and WSL.

The procedures, possible discomforts or risks, as well as possible benefits were explained fully to parents or caregivers of the children involved. After their informed consent form was signed by the parent or guardian, the children received an oral examination by the calibrated examiner, using mouth mirrors and the knee-to-knee technique²⁵⁾. The exam data included date of birth, teeth present, carious lesions present and sites, incipient lesions present and sites and restored or missing teeth or surfaces. Data were recorded onto a standardized data collection form for subsequent entry into a computer spreadsheet.

The National Institutes of Dental and Craniofacial Research dental caries diagnostic criteria, was used in the assessment of the number of decayed and filled (or crowned) primary teeth or surfaces²⁶⁾. Teeth prematurely lost due to caries and/or teeth indicated for extraction were also recorded. A child was considered to have ECC if one or more tooth surfaces were decayed, as suggested by Drury et al.²⁷⁾.

2. Sampling and Culture Procedures

A pooled plaque specimen was collected on 2 cotton swabs. One was rubbed across facial and lingual surfaces of maxillary and mandibular anterior teeth and the other across occlusal surfaces of maxillary and mandibular molars. Both swabs were placed in 1.0 ml of saline in a screw-capped vial for quantitative recovery of MS and LB. A stimulated saliva sample was collected by having the child chew for 1 to 2 minutes on half a sterile cotton roll attached to dental floss (to prevent swallowing of the cotton roll). When the cotton roll was saturated it was placed in the barrel of a sterile 5 ml disposable syringe and saliva was expressed into a sterile screw-capped vial.

The samples were processed immediately in the anaerobic microbiology laboratory at the University of Maryland Dental School. The vials containing the plaque and saliva specimens were dispersed by vortexing for 10 seconds, serially diluted (1:10) in saline (0.85% sodium chloride solution) and plated in duplicate on the following agar media: Mitis Salivarius agar (Difco, Detroit, MI) supplemented with Kanamycin and Bacitracin²⁸⁾ for MS, Rogosa SL agar (Difco) for LB, Trypticase Soy agar (BBL, Cockeysville, MD) for total viable counts (TVC). Dilution and plating were conducted by micro-techniques described by Westergren and Krasse²⁹⁾.

Inoculated plates were incubated at 37°C in air containing 10% CO₂ for 72 hours. After incubation, colony forming units (CFU) on each plate were enumerated. MS and LB were identified by colony morphology with use of a stereo microscope, and Gram stain. Counts were conducted by one investigator who had no knowledge of the clinical status of the subject.

The salivary pH was measured, using a model 420A Orion pH meter (Orion Research Inc. Beverly, MA) with an Orion Ross semi-micro combination electrode. The accuracy of the pH meter was checked at regular intervals to ensure that the readings were correct. Buffering capacity was determined by measuring the pH change after adding 20 μ l of 0.94% lactic acid to 250 μ l of the saliva.

3. Statistical Evaluations

Microbial values were entered on data forms which did not show clinical findings of the sampled subjects. The later were entered subsequent to colony enumeration. Means and standard deviations of the levels of each microbial category were calculated. Clinical values consisted of the following: 1) subject age in months, 2) number of teeth present, 3) decayed, missing and filled surfaces (dmfs) and 4) dmfs of maxillary incisors (dmfs Mx. In). Group comparisons were made for children caries-free (CF) as compared to children with ECC (ECC), as well as for children with different levels of CFU of MS or LB. CFU values were transformed into log₁₀ values to facilitate data management.

Data analyses were performed using the program Sigma Stat 2.0 (Jandel Corporation) and included Linear regression test and Multiple linear regression test. For grouped data chi-square test and odds ratios were used to assess the magnitude of the associations and Fisher exact tests were used to assess associations involving small cell sizes. To determine the validity of the different microbiological categories in the differentiation of children with ECC from those caries free, we used sensitivity, specificity and predictive values.

III. Results

Eighty-nine children less than 3 years of age were examined. Thirty-nine children presented with early childhood caries (ECC) and 48 were caries-free (CF). Descriptive data of the population are presented in Table 1. Average dmfs of total subjects was 6.3. Mean dmfs and dmfs for maxillary incisors for ECC was 14.1 and 9.4, respectively. Mean age for total subjects was 24.4.

Mean values and standard deviations for microbial counts are presented Table 2. In each microbial category, ECC values were higher than CF. Microbial counts for each category were notably higher in plaque compared to saliva. MS values were 10- to 100-fold higher than LB by each sampling method and in each caries status group. TVC values also were higher in plaque versus saliva. Mean salivary

Table 1. Descriptive data of children aged 6 to 36 months

	Total	ECC	Caries-free
Number	87	39	48
dmfs	6.3(±12.5) ^a	14.1(±15.4)	0
dmfs Mx. In	4.2(±6.9)	9.4(±7.5)	0
Age, months	24.4(±7.5)	28.2(±5.3)	21.3(±7.7)
Teeth present	15.6(±4.9)	18.0(±2.9)	13.7(±5.4)

a. mean (standard deviation)

Table 2. Microbiological findings and pH measurements according to caries status

	Total	ECC	Caries-free
MS			
Saliva/ml (×10 ⁴)	20(±42) ^a	34(±48)	8(±32)
Plaque/ml (×10 ⁴)	105(±265)	179(±247)	46(±266)
LB			
Saliva/ml (×10 ³)	7(±32)	13(±47)	2(±9)
Plaque/ml (×10 ³)	44(±167)	70(±185)	23(±150)
TVC			
Saliva/ml (×10 ⁶)	12(±24)	18(±33)	7(±12)
Plaque/ml (×10 ⁶)	25(±36)	36(±49)	16(±16)
Salivary pH	6.6(±0.7)	6.6(±0.7)	6.5(±0.7)
Δ pH with lactic acid ^b	2.3(±0.5)	2.3(±0.5)	2.2(±0.5)

a. mean (standard deviation)

b. salivary pH minus salivary pH after adding 0.94% lactic acid

pH measurements, pH drop values after adding 0.94% lactic acid were roughly similar in total, ECC and CF subjects.

Univariate associations between cross-sectional caries status (dmfs) and microbial counts were evaluated by the Linear Regression test (microbial counts were the independent variable and dmfs, the dependent variable) and shown in Table 3. The association was highly significant for MS, LB, and TVC. Salivary pH and pH drop values after adding 0.94% lactic acid versus caries status were not significant.

Table 4 shows the statistical comparisons of dmfs versus selected levels of counts of several microbial categories using caries or no caries as the outcome classifications, and specific levels of MS or LB in each specimen as risk classifications. The following risk factor values (specific microbial counts) above which caries presence is predicted, or below which

Table 3. Linear regression analysis of association between caries status and microbiological values

	Versus dmfs	Versus dmfs Mx. In
MS		
Saliva/ml	0.001 ^a	0.001
Plaque/ml	0.001	0.001
LB		
Saliva/ml	0.001	0.001
Plaque/ml	0.001	0.001
TVC		
Saliva/ml	0.001	0.001
Plaque/ml	NS ^b	0.001
MS plus LB^c		
saliva	0.001	0.001
plaque	0.001	0.001
Salivary pH		
Salivary pH	NS	NS
Δ salivary pH w/ lactic acid ^d	NS	NS
Teeth present		
Teeth present	NS	0.03
Age (months)	0.001	0.001

a. probability value

b. NS - not significant

c. multiple regression analysis

d. salivary pH minus salivary pH after adding 0.94% lactic acid

caries is not predicted were as follows: 1) saliva: 10⁵ MS/ml, 2) plaque: 2×10⁵ MS/ml, 3) saliva: 10³ LB/ml, 4) plaque: 10³ LB/ml. Odds ratios for these values ranged from 9 to 50 and Chi-square tests were all highly significant (p=0.001).

Sensitivity, specificity as well as positive and negative predictive values for associating presence or absence of dental caries at specific microbial levels, are shown in Tables 5. Sensitivity values ranged from 39% for saliva LB counts, to 82% for plaque MS counts. Specificity values were fairly high and ranged from 90% to 96%. Many microbial categories demonstrated either high specificity or sensitivity, or high positive or negative predictive values, but usually not both. Only MS at 2×10⁵/ml of plaque was associated with relatively high sensitivity and specificity and predictive values.

Table 4. Chi square and odds ratio analysis of microbial counts/ml versus caries or no caries

Counts/ml	Caries (+)	Caries (-)	Chi-square (P)	Odds Ratio
Saliva				
MS $\geq 10^5$	26	3	0.001	30
MS $< 10^5$	13	45		
LB $\geq 10^3$	15	2	0.001	15
LB $< 10^3$	23	46		
Plaque				
MS $\geq 2 \times 10^5$	32	4	0.001	50
MS $< 2 \times 10^5$	7	44		
LB $\geq 10^3$	20	5	0.001	9
LB $< 10^3$	19	43		

IV. Discussion

Early Childhood Caries (ECC), a dental caries syndrome which affects many children shortly after eruption of incisor teeth, is present in high proportions of a child 3 years old or younger³⁰⁾ and is caused by MS that ferment dietary carbohydrates to produce acid attacks on susceptible teeth over a period of time³¹⁻³³⁾.

MS are the major organisms causing the inception of primary carious lesions and primarily colonize the teeth and the presence of MS is associated with the initiation as well as the progression of dental caries³⁴⁻³⁶⁾. The relationship between the establishment of MS and the initiation of dental caries in young children has been extensively studied. Early colonization of MS increases the risk of early caries development and caries experience and/or mutans streptococcal levels in primary dentition may also be indicators of caries in permanent dentition^{7,10,37,38)}. Caufield et al.⁴⁾ reported that most colonization takes place between 19 and 31 months, a period he termed the "window of infectivity". However, some studies have found colonization of MS in the oral cavity at younger ages^{20,39,40)}.

LB are primarily colonizers of the tongue and their numbers reflect total carbohydrate consumption³⁶⁾. LB may play a significant role only during the initiation of a low percentage of coronal caries lesions but may be more important in their progression^{9,41)}. LB as well were found to be useful microbial indicators of caries, either as independent values or combined

Table 5. Sensitivity, specificity, positive and negative predictive values of specific microbial counts versus caries or no caries

Risk Category (Count/ml)	Sensitivity (%)	Specificity (%)	Positive Predictive value (%)	Negative Predictive value (%)
Saliva				
MS $\geq 10^5$	67	94	90	78
LB $\geq 10^3$	39	96	88	67
Plaque				
MS $\geq 2 \times 10^5$	82	92	89	86
LB $\geq 10^3$	51	90	80	69

with MS. The only disadvantage was their lower prevalence compared to MS. Other investigators concluded that combination of MS plus LB showed higher correlation with caries than either alone⁴²⁻⁴⁴⁾.

Stimulated saliva is generally considered to be the preferred site for microbial sampling in adults and older children because it represents the microbiota of the entire mouth and correlated well with plaque samples, for levels of MS and LB^{8,9)}. Salivary concentrations of MS $\geq 2 \times 10^5$ /ml have been accepted as indicative of high caries risk while concentrations $< 10^5$ /ml indicate low risk and for LB, concentrations $\geq 10^3$ /ml saliva represented high caries risk and $< 10^3$ /ml, low caries risk^{8,9,45)}.

Counts of MS/ml or LB/ml in stimulated saliva above which caries presence is predicted, or below which, not predicted (10^5 and 10^3 for MS and LB, respectively; Table 4), differed from that reported for adults and older children in previous investigations (2×10^5 for MS, and 10^5 for LB)^{8,9)}. Our data indicated that 10^5 MS/ml of saliva, half that of the adult value, appeared to be the best caries indicator value for saliva in our population. Differences may be attributed to fewer teeth present and reduced oral exposure of teeth in young versus older populations.

Dental plaque specimens collected with swabs then dispersed in 1.0 ml saline proved beneficial from several standpoints: 1) ease of collection and patient acceptance, 2) yield of high counts of target bacteria and 3) it was the site most likely to harbor cultivable levels of target bacteria. The plaque value for MS indicative of cariogenicity was $\geq 2 \times 10^5$. Plaque samples

recovered more MS and LB compared to saliva. Plaque sampling has the advantage over stimulate saliva, as we learned from our experience, in being easier to collect and well tolerated by the young subjects. Plaque values were not comparable to previous investigations because identical sampling and culture methods were not used. Our study also agreed with those recommending inclusion of MS with LB as a variable (Table 3), however use of single microbial counts also showed high degree of significance.

The total viable count was included to provide an indication of oral microbial density or load which might also indicate poor oral cleansing practices or stagnant saliva flow. Salivary TVC significantly correlated with dmfs, but plaque showed non-significant correlations. TVC did not appear to be useful as a risk factor.

The absence of significant association between salivary pH or buffer capacity measurements and caries status coincides with the reports by Klock and Krasse⁴⁸⁾ who found no association between caries status and salivary buffering capacity, flow rate or pH in 9- to 12-year olds, and with the results by Ansai et al.¹²⁾ who reported a non-significant correlation between caries status and salivary buffering capacity in 1- to 6-year olds. It differs, however, from the study of Grindejord et al.¹¹⁾ who found an association between salivary buffering and caries risk in a population of 2.5-year olds.

Our findings that detection of MS and, or LB are significant risk factors for presence of dental caries agreed with most previous studies of children less than 3 year old. In this study, 70% of children harboured MS which is higher than that reported by Fujiwara et al. (39.9%, aged 0-2 yr old), Grindejord et al. (28%, 2.5 yr old, 1993), Radford et al. (10.8%, 1 yr old) and Roeters et al. (43%, aged 1.9-2.8 yr old)^{10,14,47,48)}. This prevalence is less than the prevalence observed in Mattos-Graner, et al.^{17,49)} (80.3%, 1-2.5 yr old, 1998; 82.2%, 2001). Additionally, our LB colonization rate of 41% was in contrast to the results reported by Grindejord et al. (22%, 2.5 yr old), Ollila et al. (18%, aged 1-4 yr old), Radford et al. (4.6%) and Roester et al. (11.5%)^{13,14,47,50)}. The caries frequency in our project (44.8%) was higher than Grindejord's⁴⁷⁾ 2.5 year-old population with a frequency of only 6.4%. Our population was mainly a

low socio-economic group from the inner city of Baltimore which was similar to populations investigated by Mohan et al. and Karn et al.^{18,39)}. The latter also found very high colonization by MS.

Although cariogenic levels of MS were present in most subjects with caries, they were also present in many (22/48) without caries. A possible explanation is that MS may achieve relatively high levels on dental surfaces before cavitation can be detected clinically. There is abundant evidence that the latter may be true both in adults and older children^{8,9)}, and in young children⁵¹⁾, but high MS on pre-cavitated teeth usually were associated with subsurface decalcifications rather sound surfaces. Two researchers, however, found simple colonization of infants mouths at an early age by MS (either high or low levels) to be a risk factor for caries development by the 4 year old^{6,7)}. Other longitudinal investigations of young children also found presence of MS, along with other factors, to be predictive of future caries^{9,10,14,21)}. Fujiwara et al.¹⁰⁾ concluded that MS correlated more closely with caries at the subsequent year than with caries at the first sampling. In our project, 22 of the 48 CF (and incipient-free) subjects harbored MS in plaque (average count = 10^6).

Recent scientific articles have challenged or expanded conventional concepts about the microbiology of ECC. A cultural investigation of 14 caries-active (CA) and 15 caries-free children (CF), aged 2 to 8 years by Marchant et al.²²⁾, in addition to confirming the significance of MS and LB as caries risk factors, found plaque levels of *Veillonella spp.*, *Candida albicans* and *Actinomyces israelii* to be significant caries risk factors. Culture-independent microbiological identification studies are based on molecular techniques. These have the advantages over culture-dependent methods in being independent of specimen viability (e.g. specimens can be frozen) and able to recognize non-cultivable species. Genetic techniques will eventually be more rapid than cultural ones, but presently require processing in a laboratory. Tanner et al.⁴⁰⁾ using DNA probes found early colonization by *S. mutans* in children younger than 19 months. Becker et al.⁵²⁾ utilizing molecular methods to identify and quantify 23 bacterial species in plaque or carious dentin of 2- to 8-year olds reported that *Actinomyces gerencseriae* correlated most closely

with the caries status, followed by a species of *Bifidobacterium* and then *Streptococcus mutans*. These findings suggest that non-conventional species may be important in the determinations of caries risk and should be considered in future studies.

The benefit that may be obtained from this study is the enhancement of cultural measurements which may determine whether a child is at risk for caries at a very young age, and then to prevent it from occurring. A follow-up study would be helpful in monitoring the children who have been sampled and exhibit high levels of cariogenic microorganism.

V. Conclusions

To evaluate microbiological data and salivary measurements from clinically compatible, culture-based microbiological screening procedures employed with children younger than 36 months old. Plaque and stimulated saliva specimens were collected from 87 children. The pH of each saliva sample was measured before and after 0.94% lactic acid was added. After specimens were diluted and plated on selective and non-selective media, resulting bacterial counts were compared to caries status of subjects.

According to this study, the results were as follows:

1. Highly significant correlation with caries rates were found for counts of MS and LB.
2. The specific counts/ml saliva or plaque above which caries is predicted, or below which caries is not predicted were as follows:
 - 1) Saliva MS: 10^5
 - 2) Plaque MS: 2×10^5
 - 3) Saliva LB: 10^3
 - 4) Plaque LB: 10^3
3. Salivary pH and buffering capacity versus caries status were not significant.
4. Microbial screening methods based on mutans streptococci had higher predictive values and odds ratios than methods for lactobacilli.
5. MS counts were clearly the best indicators of caries status in young children. This measurement can easily be obtained in a dental clinical setting both by conventional culture techniques, or commercial kits for MS recovery.

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Abstract

**CARIES-RELATED MICROBIOLOGICAL SCREENING IN CHILDREN
UNDER THREE YEARS OF AGE**

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To evaluate microbial data and salivary measurements from clinically compatible, culture-based screening procedures employed with children younger than 36 months old. Plaque and stimulated saliva specimens were collected from 87 children. The pH of each saliva sample was measured before and after 0.94% lactic acid was added. Specimens were diluted and plated on selective media and non-selective media. Data collected were counts of mutans streptococci (MS) and lactobacilli (LB). In addition, total viable counts (TVC) of specimens, salivary pH and buffering capacity were also assessed. Each variable was compared to caries status of subjects.

According to this study, the results were as followed:

1. Highly significant correlation with caries rates were found for counts of MS and LB.
2. The specific counts/ml saliva or plaque above which caries is predicted, or below which caries is not predicted were as follows:
 - 1) Saliva MS: 10^5
 - 2) Plaque MS: 2×10^5
 - 3) Saliva LB: 10^3
 - 4) Plaque LB: 10^3
3. Salivary pH and buffering capacity versus caries status were not significant.
4. Microbial screening methods based on mutans streptococci had higher predictive values and odds ratios than methods for lactobacilli.
5. MS counts were clearly the best indicators of caries status in young children. This measurement can easily be obtained in a dental clinical setting both by conventional culture techniques, or commercial kits for MS recovery.

Key words : Plaque, Stimulated saliva, Mutans streptococci, Lactobacilli, Salivary pH, Buffering capacity