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Microbiology in toothbrush samples from children exposed to lead in southern Thailand

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Abstract

Objective. The purpose of this study was to evaluate the prevalence and level of selected oral bacterial species in association with dental caries in low versus high blood lead (PbB) children. ***Material and methods***. With an observational cross-sectional design, a sample of 292 children aged 6–11 years from two primary schools around a shipyard, known to be an area contaminated with lead, were examined. The number of decayed and filled surfaces on deciduous teeth (dfs) and the number of decayed, missing, and filled surfaces on permanent teeth (DMFS) were recorded. Microbiological plaque samples were taken from each child with a toothbrush. Enumeration of 17 bacterial species was carried out using the checkerboard DNA-DNA hybridization technique. ***Results***. Overall means (SD) of dfs and DMFS were 13.2 (9.5) and 1.3 (2.3), respectively. Prevalence of excessive count ($\geq 10^6$ cells/sample) was 100% for 4 known cariogenic bacteria and over 95% in another 2 species. With Bonferroni correction for type I error adjustment, there was no significant association between the count of each bacterial species and PbB and caries experience. The checkerboard method is sensitive in bacteria detection, but may not be suitable for differentiating caries risk in the endemic population.

Key Words: *Blood lead, children, dental caries, oral microbiology*

Introduction

Lead (Pb) is a major environmental pollutant and a hazard to health, and the adverse health effects of lead exposure in children are well documented [1,2]. One of its main targets is the nervous system [2]. Meta-analysis has revealed a highly significant association between blood lead (PbB) levels and IQ in school-aged children [3]. An association between lead exposure and higher caries prevalence has also been suggested, but even the few published studies so far have shown controversial results [4–7]. Our previous report demonstrated a significantly higher caries in deciduous teeth in high PbB compared to low PbB children [8]. However, the mechanisms behind lead influence on caries development remain unclear. Mineralization of the enamel can be altered due to a delay in enamel maturation [8,9], and this may increase susceptibility of the tooth to the dental caries process. However, no morphological changes connected with lead in blood have been found in the deciduous teeth of these children [10]. Secondly, administration of lead can significantly diminish (30–40%) stimulated salivary flow rates [11] and this may have an indirect influence on bacterial activity and plaque formation rate. Thirdly, lead may also have a direct effect on bacteria, an effect that may result in alterations in the activity of some bacterial species and subsequently the composition of the oral microflora. Schamschula et al. (1978) [12] found that elevated levels of lead in plaque were associated with increased prevalence of caries; however, the microflora was not studied. Gil et al. (1996) [6] documented high numbers of salivary lactobacilli and mutans streptococci in a group of individuals with high lead level in teeth. For a better relevance to caries than saliva, it has been suggested that assessment of lactobacilli, mutans streptococci, and other bacterial species should be determined in the dental plaque [13].

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Using the DNA-DNA checkerboard method, the purpose of this study was to evaluate the prevalence and level of selected oral bacterial species in the dental plaque of high PbB children compared to low PbB children. Species were collected as a pooled sample using a toothbrush. Furthermore, it was intended to study whether the bacteria were associated with dental caries in both groups of lead-exposed children.

Material and methods

Study setting

The study area was a shipyard which had been environmentally lead contaminated due to the use of Pb_3O_4 in the ship-repair industry of Huakhao subdistrict, Songkhla Province, Southern Thailand. The Ethics Committee, Prince of Songkla University, approved the study protocol.

Subjects

Schoolchildren, aged 6–11 years and enrolled in the 2002 academic year of two primary schools (Banbosub and Banhuakao) located in the area, were enlisted. Only children with no major health problem and having resided in the area since birth were invited to participate in the study. The parents signed the agreement consent forms before data were collected.

PbB

Four milliliters of venous blood was drawn from the cubital vein of the subject and sent for analysis of PbB level using a Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS, HITACHI Model Z-8200) at the Faculty of Tropical Medicine, Mahidol University, Bangkok. The children with PbB >10 μg/dl were considered as highly lead-exposed according to the Centers for Disease Control and Prevention [14].

Oral hygiene

The Simplified Oral Hygiene Index [15] was assessed by inspection of facial surfaces of tooth numbers 16, 11, 26, 31, and the lingual surfaces of tooth numbers 36 and 46. It consisted of the debris index and calculus index. The plaque index was classified as mild, moderate, or heavy, and the calculus index as present or absent.

Dental caries

Clinical oral examination was conducted to define carious lesions and oral status in accordance with the criteria of the World Health Organization (1997) [16]. The number of decayed and filled surfaces on deciduous teeth (dfs) and the number of decayed, missing, and filled surfaces on permanent teeth (DMFS) were determined. All examinations were carried out by the one dentist with an intra-examiner kappa statistic of 0.96. The examiner was blinded to children's PbB during the examination.

Bacterial plaque samples

Plaque samples were collected by the toothbrush method [17]. A new toothbrush (6 to 9-year-old size, St. Andrews, Sahapattanapibul Ltd., Thailand) was used for each child. The children's teeth were brushed over all erupted teeth. The toothbrush was then dripped into 15 ml distilled water and vigorously shaken to obtain the plaque suspension sample. The sample was centrifuged at 5,000 rounds per minute (rpm) for 10 min. Then 150 μl of sterile TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) was added to the plaque pellet and the bacterial suspension was transferred to an Eppendorf tube and stored at -20°C until further processing.

Checkerboard DNA-DNA hybridization

The analysis of bacterial species was performed using the checkerboard DNA-DNA hybridization method [18,19] at the laboratory of Oral Microbiology, Göteborg University, Sweden. Digoxigenin-labeled, whole genomic DNA probes were prepared using the High-Prime labeling kit (Boehringer-Mannheim, Germany). Genomic DNA of bacterial species were *Streptococcus mutans* (ATCC 25175), *Streptococcus sanguinis* (ATCC 10566), *Streptococcus oralis* (ATCC 35037), *Streptococcus intermedius* (ATCC 27335), *Lactobacillus acidophilus* (ATCC 4356), *Veillonella parvula* (ATCC 10790), *Actinobacillus actinomycetemcomitans* (FDCY4), *Eikenella corrodens* (ATCC 23834), *Campylobacter rectus* (ATCC 33238), *Fusobacterium nucleatum* (ATCC 10953), *Selenomonas noxia* (OMGS 3118), *Capnocytophaga ochracea* (ATCC 33624), *Prevotella intermedia* (ATCC 25611), *Prevotella nigrescens* (ATCC 33563), *Porphyromonas gingivalis* (FDC 381), *Tannerella forsythia* (formerly *Bacteroides forsythus* ATCC 43037), *Actinomyces naeslundii* genospecies 2 (ATCC 15987). The samples were boiled for 5 min, neutralized, laid down on nylon membranes by means of a blotting device (Immunetics, Cambridge, Mass., USA), and immobilized by UV light for 45 s and incubated at 120°C for 20 min. After 2 h of prehybridization, the standard DNA probes were allowed to hybridize overnight with the sample DNA using a second blotting device (Immunetics, Cambridge, Mass., USA) at 42°C. After low and high stringency washes at 65°C, hybrids were detected by application of an anti-digoxigenin antibody conjugated with alkaline phosphatase and incubation with an appropriate chemiluminescent substitute (CSPD,

Boehringer Mannheim). Evaluation of the number of bacteria in the samples was performed by comparing the obtained signals with the ones generated by pooled standard samples containing 10^6 and 10^5 of each species. The signals were coded on a scale from 0 to 5, with 0 indicating no signal; 1, a signal density weaker than that of the low standard (i.e. $<10^5$ bacteria); 2, a signal density equal to that of the low standard ($=10^5$ bacteria); 3, a signal density higher than that of the low standard but lower than that of the high standard ($>10^5$ but $<10^6$ bacteria); 4, a signal density equal to that of the high standard ($=10^6$ bacteria); 5, a signal density higher than the one of the high standard ($>10^6$ bacteria). The assessment was performed by one examiner.

Data analysis

Double entry of data was carried out using the Epidata 2.1 program ("The EpiData Association", Odense, Denmark) to validate the accuracy of entering data. Stata 7.0 was used for statistical analysis. The number of bacterial cells was re-categorized into three groups: $\leq 10^5$, 10^5–10^6, and $\geq 10^6$ cells. For comparison between dfs and DMFS and the level of bacteria in high PbB and low PbB children, the data were dichotomized ($<10^6$ and $\geq 10^6$ cells). Breakdown of means of dfs and DMFS was carried out by level of each bacterial count ($<10^6$ and $\geq 10^6$ cells) and stratified by PbB level (<10 μg/dl vs ≥ 10 μg/dl). Initially, the mean and standard error (SE) of dfs and DMFS were displayed. The *t*-test result was used to examine the effect of bacterial count on dfs and DMFS in each stratum. Multiple linear regression was then used to adjust for the effect of potential confounders, i.e. age, gender, socio-economic status, and oral health behaviors. Finally, adjusted means of the overall subjects were presented based on the regression models.

Since there were 17 bacterial species being tested simultaneously for association with caries, Bonferroni correction for type I error adjustment was used for all hypothesis testing related to bacteria in this article [20]. Significant value instead of 0.05 was $1-(1-0.05)^{1/17}$ or 0.003, while highly significant value was $1-(1-0.01)^{1/17}$ or 0.0005.

Results

Two-hundred-and-ninety-two children aged 6–11 years completed clinical oral examination, PbB level, and bacterial determination. Twenty children had only permanent teeth and three had only deciduous teeth. Therefore, 272 children remained for analysis of deciduous teeth and 289 children for analysis of permanent teeth. Important characteristics and clinical parameters of subjects are described in Table I. The two groups of lead-exposed children had similar oral hygiene and total number of present tooth surfaces. The mean dfs number was significantly higher in high PbB children than in low PbB children ($p<0.01$), whereas the mean DMFS was not significantly different between the two groups.

The distribution of bacteria at different levels in the two groups of children can be seen in Table II. High prevalent and predominating species as shown by the high percentage detection of $\geq 10^6$ cells included *A. naeslundii* (100%), *S. sanguinis* (100%), *S. mutans* (100%), *S. intermedius* (99%), *S. oralis* (98%), *E. corrodens* (96%), *C. ochracea* (89%), *P. nigrescens* (68%), and *P. intermedia* (52%). Low prevalent species in the predominant flora included *V. parvula* (24%), *A. actinomycetemcomitans* (15%), *F. nucleatum* (10%), *L. acidophilus* (2%), *P. gingivalis* (1%), *T. forsythia* (0.3%), *S. noxia* (0.3%), and *C. rectus* (0%). Comparing the bacterial distribution between the two groups of lead exposure in Table II, none of the differences of each bacteria tested between the low and high PbB groups reached statistical significance. A crude analysis in Table III indicates a significant association only between dfs and *V. parvula* among children with low PbB ($p<0.003$). After adjustment for PbB level, the difference disappeared. No bacterial species was significantly associated with mean dfs number in high PbB group.

For permanent teeth (Table IV), no bacterial species was significantly associated with a mean DMFS number in either the low or the high PbB groups, nor in the adjusted analysis.

Discussion

The results of this investigation indicate a wide range and high quantity of oral bacteria in children in this population of southern Thailand. Of all species investigated, none showed an association with PbB level after Bonferroni correction adjustment. With regard to dental caries in the deciduous dentition, a high level of *V. parvula* was associated with high dfs in low PbB children. The difference disappeared in adjusted analysis. For dental caries in the permanent dentition, no bacterial species was significantly associated with DMFS.

The finding of high bacterial levels for some of streptococcal species and *Actinomyces naeslundii* in this study is not surprising in view of the collected plaque being supragingival [21]. The use of toothbrush samples instead of sampling plaque with a curette probably overloads the sample with bacteria and the upper detection limit is exceeded in the checkerboard DNA-DNA assay. Prevalence of excessive count ($\geq 10^6$ cells/sample) was 100% for 4 known cariogenic bacteria and over 95% in another 2 species. The checkerboard assay also contributes, as do most molecular based methods, to an increase in prevalence for the majority of species as compared

Table I. Characteristics and clinical parameters of the test children

Variable	All children ($n=292$) n (%) or mean [SD]	Children with blood lead level <10 μg/dl $n=232$	Children with blood lead level ≥ 10 μg/dl $n=60$
Age (years)	8.8 [1.2]	8.8 [1.3]	8.8 [1.1]
Gender			
Boy	143 (49)	116 (50)	27 (45)
Girl	149 (51)	116 (50)	33 (55)
Tooth surface present	100 [7.9]	101.3 [7.6]	98.7 [8.9]
Permanent surface present	57.6 [22.8]	58.1 [23.6]	55.7 [19.3]
Deciduous surface present	43.1 [22.8]	43.2 [23.3]	43.0 [21.2]
DMFS	1.3 [2.3]	1.2 [2.3]	1.6 [2.4]
dfs*	13.2 [9.5]	12.3 [8.9]	16.5 [11.2]
Plaque			
Mild	55 (19)	47 (20)	8 (13)
Moderate	139 (48)	110 (48)	29 (48)
Heavy	98 (33)	75 (32)	23 (39)
Calculus			
No	179 (61)	144 (62)	35 (58)
Mild	83 (29)	63 (27)	20 (33)
Moderate and heavy	30 (10)	25 (11)	5 (9)

*Statistically significant difference between the two groups of children: p-value <0.01.

to the conventional culture technique, particularly fastidious periodontal pathogens [19].

Both *P. gingivalis* and *T. forsythia* were detected less frequently by culture than either with immunofluorescence or with the checkerboard technique [22]. The quality of the template DNA may also affect the sensitivity and specificity of the findings [23]. A lower specificity of the checkerboard technique can occur in heavy plaque samples [19] and in this respect the toothbrush samples should be avoided in patients with poor oral hygiene and high plaque scores. Thus, statistical calculations were not performed on *A. naeslundii*, *S. mutans*, *S. sanguinis*, and *S. intermedius* due to high prevalence and levels generally over 10^6.

Recorded bacteria in our study included early plaque colonizing species such as *S. sanguinis*, *S. oralis*, and *Actinomyces* spp., which could be found in the oral cavity of children as early as infancy or predentate period [24,25]. These play an important part in supragingival plaque formation on both deciduous and permanent teeth [26]. Other organisms generally regarded as late colonizers [26], such as *Fusobacterium* spp., *Prevotella* spp., *E. corrodens*, and *C. ochracea*, are reported to be common in children [27–29], and the high prevalence of these

Table II. Distribution of bacterial count from toothbrush sample by PbB level of the study subjects

Bacterial species	Children with PbB <10 µg/dl (n (%))			Children with PbB ≥ 10 µg/dl (n (%))			p-value
	$\leq 10^5$	10^5–10^6	$\geq 10^6$	$\leq 10^5$	10^5 –10^6	$\geq 10^6$	
A. naeslundii	– (0)	– (0)	232 100)	– (0)	– (0)	60 (100)	NA
S. sanguinis	– (0)	– (0)	232 (100)	– (0)	– (0)	60 (100)	NA
S. mutans	– (0)	1 (1)	231 (99)	– (0)	– (0)	60 (100)	0.610
S. intermedius	– (0)	2 (1)	230 (99)	– (0)	– (0)	60 (100)	0.470
S. oralis	– (0)	6 (3)	226 (97)	– (0)	1 (2)	59 (98)	0.678
E. corrodens	– (0)	10 (4)	222 (96)	– (0)	1 (2)	59 (98)	0.338
C. ochracea	14 (6)	15 (6)	203 (88)	– (0)	4 (7)	56 (93)	0.149
P. nigrescens	22 (9)	55 (24)	155 (67)	5 (8)	11 (18)	44 (74)	0.614
P. intermedia	20 (9)	89 (38)	123 (53)	4 (7)	26 (43)	30 (50)	0.740
V. parvula	4 (2)	181 (78)	47 (20)	1 (2)	36 (60)	23 (38)	0.014
A. actinomycetemcomitans	3 (1)	195 (84)	34 (15)	1 (2)	48 (80)	11 (18)	0.755
F. nucleatum	2 (1)	212 (91)	18 (8)	0 (0)	50 (83)	10 (17)	0.090
L. acidophilus	17 (7)	209 (90)	6 (3)	13 (22)	46 (77)	1 (2)	0.005
P. gingivalis	130 (56)	101 (43)	1 (1)	30 (50)	29 (48)	1 (2)	0.444
S. noxia	56 (24)	175 (75)	1 (1)	12 (20)	48 (80)	– (0)	0.691
T. forsythia	85 (37)	146 (63)	1 (0)	19 (32)	41 (68)	– (0)	0.668
C. rectus	186 (80)	46 (19)	– (0)	53 (88)	7 (12)	– (0)	0.144

NA denotes not available due to the fact that all samples had $\geq 10^6$ cells.

Table III. Mean (SE) of dfs in relation to different bacterial levels in low and high PbB children ($n = 272$)

	dfs					
	Low PbB children		High PbB children		All children[c]	
Bacteria	$< 10^6$	$\geq 10^6$	$< 10^6$	$\geq 10^6$	$< 10^6$	$\geq 10^6$
A. naeslundii	NA	NA	NA	NA	NA	NA
S. sanguinis	NA	NA	NA	NA	NA	NA
S. mutans	NA	NA	NA	NA	NA	NA
S. intermedius	NA	NA	NA	NA	NA	NA
S. oralis[b]	10.25 (2.17)	12.35 (0.62)	19 (–)	16.46 (1.52)	11.82 (4.20)	13.18 (0.57)
E. corrodens[b]	17.20 (3.80)	12.07 (0.61)	28 (–)	16.30 (1.50)	18.29 (2.81)	12.94 (0.58)
C. ochracea	12.00 (1.48)	12.35 (0.66)	22.00 (6.82)	16.09 (1.53)	13.69 (1.74)	13.09 (0.60)
P. nigrescens	11.29 (1.15)	12.79 (0.71)	16.47 (2.92)	16.52 (1.76)	12.40 (1.02)	13.49 (0.68)
P. intermedia	11.50 (0.86)	13.01 (0.85)	13.96 (1.68)	18.8 (2.35)	12.04 (0.83)	14.13 (0.77)
V. parvula	11.35⋆ (0.65)	16.02⋆ (1.43)	15.67 (1.91)	18.05 (2.39)	12.30 (0.64)	15.94 (1.18)
A. actinomycetemcomitans	12.37 (0.66)	11.93 (1.58)	16.37 (1.66)	17.22 (3.53)	13.24 (0.62)	12.68 (1.45)
F. nucleatum	11.97 (0.63)	15.94 (2.12)	15.62 (1.52)	21.22 (4.83)	12.74 (0.59)	16.93 (1.80)
L. acidophilus[a]	10.61 (2.29)	12.41 (0.63)	13.42 (2.23)	17.33 (1.78)	11.20 (1.88)	13.35(0.60)
P. gingivalis[a]	11.29 (0.80)	13.52 (0.93)	19.00 (2.29)	14.27 (1.89)	12.86 (0.78)	13.48 (0.83)
S. noxia[a]	10.22 (1.22)	12.96 (0.70)	17.36 (3.37)	16.30 (1.68)	11.58 (1.19)	13.62 (0.64)
T. forsythia[a]	11.05 (0.99)	12.99 (0.77)	16.72 (2.79)	16.41 (1.79)	12.41 (0.97)	13.55 (0.70)
C. rectus[a]	12.25 (0.71)	12.53 (1.11)	16.30 (1.57)	18.00 (4.91)	13.10 (0.63)	13.39 (1.30)

⋆Statistically significant difference at p-value < 0.003 within the low PbB level.
[a]Strains were categorized into $\leq 10^5$ and $> 10^5$ cells.
[b]Strains were not tested for difference in high PbB group due to too high percentage of $\geq 10^6$ cells.
[c]Adjusted for PbB.
NA denotes mean and SD not available due to the fact that all samples had $\geq 10^6$ cells.

bacteria in this study is not surprising. Bacterial species such as *P. gingivalis*, *T. forsythia*, and *A. actinomycetemcomitans*, which are associated with periodontitis, were also detected in almost all children in the present study, although at a lower level. A prevalence higher than previously thought has also been recorded for these bacteria using molecular biology techniques [30]. Conclusively, no apparent difference was found in the dental plaque flora in these children from southern Thailand compared with what could be expected from other populations.

Bacteria such as mutans streptococci and lactobacilli are known to be associated with caries and

Table IV. Mean (SE) of DMFS in relation to different bacterial levels in low and high PbB children ($n = 289$)

	DMFS					
	Low PbB children		High PbB children		All children[c]	
Bacteria	$< 10^6$	$\geq 10^6$	$< 10^6$	$\geq 10^6$	$< 10^6$	$\geq 10^6$
A. naeslundii	NA	NA	NA	NA	NA	NA
S. sanguinis	NA	NA	NA	NA	NA	NA
S. mutans	NA	NA	NA	NA	NA	NA
S. intermedius	NA	NA	NA	NA	NA	NA
S. oralis[b]	2.17 (1.60)	1.17 (0.15)	2.00 (–)	1.54 (0.32)	2.14 (0.88)	1.24 (0.14)
E. corrodens[b]	0.80 (0.36)	1.21 (0.16)	4.00 (–)	1.51 (0.32)	1.10 (0.71)	1.27 (0.14)
C. ochracea	1.48 (0.59)	1.15 (0.15)	0 (0)	1.66 (0.33)	1.34 (0.41)	1.26 (0.15)
P. nigrescens	1.12 (0.23)	1.23 (0.20)	1.37 (0.47)	1.61 (0.40)	1.18 (0.24)	1.31 (0.17)
P. intermedia	1.38 (0.23)	1.02 (0.20)	1.87 (0.53)	1.23 (0.33)	1.48 (0.20)	1.07 (0.19)
V. parvula	1.20 (0.18)	1.14 (0.29)	1.24 (0.26)	2.04 (0.71)	1.23 (0.16)	1.39 (0.29)
A. actinomycetemcomitans	1.32 (0.17)	0.35 (0.16)	1.41 (0.27)	2.18 (1.23)	1.35 (0.15)	0.80 (0.36)
F. nucleatum	1.20 (0.16)	1.11 (0.63)	1.20 (0.23)	3.30 (1.45)	1.20 (0.14)	1.84 (0.44)
L. acidophilus[a]	2.71 (0.91)	1.07 (0.14)	1.31 (0.46)	1.62 (0.38)	2.06 (0.43)	1.17 (0.14)
P. gingivalis[a]	1.18 (0.18)	1.21 (0.26)	2.07 (0.56)	1.03 (0.26)	1.35 (0.18)	1.16 (0.21)
S. noxia[a]	1.29 (0.34)	1.16 (0.17)	1.50 (0.54)	1.56 (0.37)	1.34 (0.29)	1.24 (0.16)
T. forsythia[a]	1.14 (0.23)	1.22 (0.20)	1.84 (0.45)	1.41 (0.41)	1.29 (0.23)	1.25 (0.17)
C. rectus[a]	1.32 (0.18)	0.67 (0.24)	1.51 (0.35)	1.86 (0.70)	1.36 (0.15)	0.84 (0.32)

[a]Strains were categorized into $\leq 10^5$ and $> 10^5$ cells.
[b]Strains were not tested for difference in high PbB group due to too high percentage of $\geq 10^6$ cells.
[c]Adjusted for PbB.
NA denotes mean and SD not available due to the fact that all samples had $\geq 10^6$ cells.

indicate active caries or a high caries risk [21,26,31,32]. More caries was recorded for the deciduous teeth in the high PbB group but not for their permanent teeth (Table I). The lack of higher caries prevalence in the permanent teeth was explained by the fact that these teeth are newly erupted in the present group of children and an association between bacteria and DMFS cannot be expected in this age group [8]. On the other hand, the children had developed significantly more caries in the deciduous teeth and therefore higher levels of both mutans streptococci and lactobacilli in the high PbB group were expected. This association has also been documented for children with high PbB levels in their teeth [6]. However, no association between the dfs values in these children versus mutans streptococci and/or lactobacilli in saliva was found in the previous study [8]. Uniformly high levels of *S. mutans* in the subjects of the present study made it impossible to document the association between these bacteria and dental caries. This study showed no association between *L. acidophilus* and dfs in the high PbB group (Table III), suggesting a limited relation to caries. This perhaps unexpected finding could be explained by different methods of specimen collection (e.g. saliva versus plaque) and the specific detection of *L. acidophilus* in this study instead of the total lactobacillus counts as in the previous study [8].

The potential of toothbrush sampling and checkerboard technology is based on a simple technique of specimen collection that requires no special clinical skill and the high sensitivity of the laboratory test. However, in the endemic area for caries, several bacterial species had excessive counts, making comparison among groups of subjects impossible. The value of risk differentiation of the technique is therefore limited. A serial dilution to optimize the count distribution is worth attempting in the future study.

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