FINAL REPORT

DENTAL CARIES AND LEAD EXPOSURE IN CHILDREN

Nattaporn Youravong

Assoc. Prof. Rawee Teanpaisan

Prof. Virasakdi Chongsuvivatwong

Prof. Gunnar Dahlén

Dr. Alan F. Geater

May 2005

BGJ4580015

FINAL REPORT

DENTAL CARIES AND LEAD EXPOSURE IN CHILDREN

Nattaporn Youravong[1], Assoc. Prof. Rawee Teanpaisan[2],

Prof. Virasakdi Chongsuvivatwong[1], Prof. Gunnar Dahlén [3],

Dr. Alan F. Geater[1]

[1] Faculty of Medicine, Prince of Songkla University, Thailand

[2] Faculty of Dentistry, Prince of Songkla University, Thailand

[3] Sahlgrenska Academy at Göteborg University , Sweden

Supported by Thailand Research Fund

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Acknowledgements

This Ph.D. thesis work was carried out in Thailand and Sweden under financial supports by Thailand Research Fund (TRF) through Royal Golden Jubilee scholarship (RGJ) and Basic Research Grant for Royal Golden Jubilee Ph.D. program (BGJ). Some of this study was also supported by the research fund of the Commission on Higher Education, Ministry of Education and the Swedish Foundation for International Cooperation in Research and Higher Education (STINT). I sincerely acknowledge all the funds to my study.

I would like to express my respectful gratitude to all of my supervisors: Assoc. Prof. Rawee Teanpaisan, Prof. Virasakdi Chongsuvivatwong, Dr. Alan F. Geater, Prof. Gunnar Dahlén for appreciable advice and help throughout the thesis work.

I would like to thank all health officers, health volunteers, schoolteachers at Huakhao subdistrict, Singhanakhon District, especially to subjects and their parent for all kindness and co-operation.

My sincere thanks go to Nipa Maharachpong for her kind help in introducing me to the local community surrounding the shipyards and assisting me to collect blood samples.

I appreciate Dr. Wirat Eungpoonsawat for allowing Mrs. Pattaraporn Wangchai to be an assistant in some of fieldwork.

I would like to also thank all students and staff in Epidemiology Unit, Prince of Songkla University for their encouragement.

I am grateful to all staff in Oral Microbiology Department, Göteborg University, Sweden for their kindly help. My special thanks go to Susanne Blomqvist, Lisbeth Bengfsson, and Annette Carlson for kindly technical assistance and fully valuable advice.

I gratefully acknowledge my co-researcher, Prof. Jörgen G. Norén, Department of Pedodontics, Faculty of Odontology, Göteborg University, Sweden for his invaluable dedication in tooth analysis.

Finally, I wish to thank to my family: my father and mother, and my husband, for their encouragement, especially to my husband who encouraged me to undertake Ph.D. studies and provided me good support throughout the study years.

Nattaporn Youravong

Abstract

Project Code : BGJ4580015

Project Title : Dental caries and lead exposure in children

Investigator : Nattaporn Youravong

Epidemiology Unit, Faculty of Medicine,

Prince of Songkla University, Hatyai, Songkhla

E-mail Address : pnuttapo@yahoo.com

Project Period : 30 September, 2002 to 29 September 2004

This thesis concerns about the dental caries status of children living around shipyards, previously recognized as a potential source of lead contamination. The study area located in Huakhao subdistrict, Singhanakhon District, Songkhla Province.

The study assessed the prevalence and severity of the dental caries in children living in the area, and the association of the dental caries with blood lead exposure. Additionally, there were two subsidiary objectives. Subsidiary objective I aimed to evaluate with the DNA-DNA checkerboard method, the prevalence and level of selected oral bacterial species in dental plaque collected as a pooled sample using a toothbrush of high lead-exposed children compared to low lead-exposed children. Further, it was intended to study whether those bacteria were associated with dental caries in the two groups of lead-exposed children. Subsidiary objective II aimed to examine the morphology of primary teeth in children with high blood lead levels and compare with a group of children with low blood lead levels.

The study involved an observational cross-sectional study where 292 schoolchildren aged 6-11 years were recruited from two selected primary schools around shipyards.

The number of decayed and filled surfaces on deciduous teeth (dfs), and the number of decayed, missing, and filled surfaces on permanent teeth (DMFS), the saliva flow rate, pH, buffer capacity, oral hygiene, *Lactobacillus* spp. and mutans streptococci counts were recorded. Trained interviewers also visited the households of the subjects to interview their parent/guardians for information on their socio-economic status and oral hygiene behaviours, using structured questionnaire.

Among 292 subjects, overall the prevalence of caries was high (94% in primary teeth and 44% in permanent teeth). The mean (range) of DMFS and dfs were respectively 1.3 (range 0-17) and 13.2 (range 0-45); and the geometric mean blood lead level (PbB) and SD were 7.2 and 1.5 μg/dl (range 2.5-21.3). The prevalence of PbB $\geq$10 μg/dl was twenty-one percent. The level of dfs, but not DMFS correlated with the blood lead level (R_s = 0.25, p = 0.00/ R_s = 0.09, p=0.14). The odds ratio for DMFS $\geq$1 and dfs >5 for a doubling of PbB after adjusting for other factors were 1.28 (95% CI, 0.81-2.04; p value = 0.35) and 2.39 (95% CI, 1.36-4.20; p value = 0.004), respectively.

For subsidiary objective, microbiological plaque samples were taken from each subject with a toothbrush method. Enumeration of 17 bacterial species was carried out using the checkerboard DNA-DNA hybridization technique. *Lactobacillus acidophilus* counts were significantly lower in high lead-exposed children compared to those of the low lead-exposed group (p <0.01). The level of *Veillonella parvula* was significantly higher in children with a high blood lead level (p <0.01). *V. parvula* was also associated with high dfs in children with low PbB. Other associations between the levels of bacterial species and caries experience (DMFS and dfs) in children with high blood lead levels in comparison with those with low blood lead levels were of no or little significance.

For subsidiary objective II, the morphology of enamel of primary tooth was examined by polarized light microscopy (PLM), microradiography (MRG), and scanning electron microscopy (SEM). Eighty tooth specimens derived from two groups of children: 23 teeth from group with high blood lead levels and 57 teeth from group having low blood lead

levels. The enamel irrespective of group appeared normal. However, in a majority of the specimens, the enamel surface appeared hypomineralized, which was confirmed in SEM. No morphological changes connected to lead in blood could be found.

In conclusion, blood lead exposure was associated with dental caries after adjusting for caries-associated factors. Currently the mechanism could not be explained by its action through microorganisms, saliva parameters, and enamel morphology in this study. This research provides an additional compulsory reason for an effective program of removing lead from the area. Also lead screening should be offered to the children at the area. Boosting special caries preventive measures for oral health and public education for self-care practice should be provided.

Keywords : Lead, dental caries, children, oral bacteria, tooth

บทคัดย่อ

รหัสโครงการ : BGJ4580015

ชื่อโครงการ : โรคฟันผุในเด็กที่ได้รับสารตะกั่ว

ชื่อนักวิจัย : ณัฐพร ยูรวงศ์

หน่วยระบาดวิทยา คณะแพทยศาสตร์

มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา

E-mail Address : pnuttapo@yahoo.com

ระยะเวลาโครงการ : 30 กันยายน 2545 ถึง 29 กันยายน 2547

รายงานวิจัยฉบับนี้ได้ศึกษาถึงความชุกและความรุนแรงของการเกิดโรคฟันผุในเด็กที่อาศัยอยู่ในพื้นที่ปนเปื้อนสารตะกั่ว ต.หัวเขา อ.สิงหนคร จ.สงขลา และความสัมพันธ์ระหว่างโรคฟันผุและระดับตะกั่วในเลือด นอกจากนี้ยังได้ศึกษาเพิ่มเติมถึงระดับของเชื้อในช่องปากเปรียบเทียบระหว่างกลุ่มที่มีระดับตะกั่วในเลือดสูงและกลุ่มที่มีระดับตะกั่วในเลือดต่ำ รวมถึงความสัมพันธ์ของแบคทีเรียเหล่านี้กับโรคฟันผุด้วย งานวิจัยยังได้ศึกษาเพิ่มเติมถึงลักษณะของฟันน้ำนมเปรียบเทียบระหว่างสองกลุ่มดังกล่าวด้วย

งานวิจัยนี้เป็นการศึกษาแบบตัดขวาง ในเด็กนักเรียนอายุ 6-11 ปี จากโรงเรียน 2 แห่งที่ตั้งอยู่รอบอู่ต่อเรือ จำนวน 292 คน โดยได้ตรวจและเก็บปัจจัยต่างๆดังนี้: จำนวนฟันผุและอุดในฟันน้ำนม (dfs), จำนวนฟันผุ,ถอน,อุด ในฟันแท้ (DMFS), อัตราการไหลของน้ำลาย, ความเป็นกรดด่างของน้ำลาย,ความสามารถในการปรับสภาพความเป็นกรด-ด่างของน้ำลาย, อนามัยช่องปาก, ระดับเชื้อ Lactobacillus spp. และ เชื้อ mutans streptococci นอกจากนี้ได้สัมภาษณ์เด็กนักเรียนและผู้ปกครองหรือผู้ดูแลเด็กเกี่ยวกับสถานะทางเศรษฐกิจ,ครอบครัว, และการดูแลสุขภาพช่องปากของเด็กด้วย

จากการศึกษาพบว่า ในเด็กนักเรียน 292 คน มีความชุกของโรคฟันผุสูงมาก (94% ในฟันน้ำนม และ 44% ในฟันแท้) และมีค่าเฉลี่ย DMFS และ dfs เท่ากับ 1.3 ด้าน (มีค่า 0-17)

และ 13.2 ด้าน (มีค่า 0-45) ตามลำดับ ระดับตะกั่วในเลือดโดยเฉลี่ยเท่ากับ 7.2 ไมโครกรัมต่อเดซิลิตร (ส่วนเบี่ยงเบนมาตรฐาน=1.5, มีค่า 2.5-21.3) เด็ก 21% มีค่าระดับตะกั่วในเลือดสูงกว่าหรือเท่ากับ 10 ไมโครกรัมต่อเดซิลิตร และพบว่าระดับตะกั่วในเลือดที่สูงขึ้นสัมพันธ์กับระดับของ dfs (R_s = 0.25, p = 0.00) แต่ไม่พบความสัมพันธ์กับระดับของ DMFS (R_s = 0.09, p = 0.14) ค่า odds ratio สำหรับ DMFS $\geq$1 และ dfs >5 เมื่อ PbB เพิ่มขึ้นทุก 2 เท่า หลังจากควบคุมตัวแปรอื่นๆ เท่ากับ 1.28 (95% CI, 0.81-2.04; p value = 0.35) และ 2.39 (95% CI, 1.36-4.20; p value = 0.004) ตามลำดับ

ผลของการศึกษาเพิ่มเติมจากการเก็บคราบจุลินทรีย์เพื่อศึกษาเชื้อในช่องปากโดยวิธี checkerboard DNA-DNA hybridization technique พบว่าในกลุ่มที่มีระดับตะกั่วในเลือดสูงมีระดับเชื้อ *Lactobacillus acidophilus* ต่ำกว่ากลุ่มที่มีระดับตะกั่วต่ำอย่างมีนัยสำคัญ (p <0.01) ในขณะที่ระดับเชื้อ *Veillonella parvula* สูงกว่ากลุ่มที่มีระดับตะกั่วในเลือดต่ำ (p <0.01) เมื่อศึกษาถึงความสัมพันธ์กับโรคฟันผุพบว่าเชื้อ *Veillonella parvula* เพิ่มขึ้นสัมพันธ์กับฟันผุในฟันน้ำนมที่เพิ่มขึ้น ในกลุ่มที่มีระดับตะกั่วในเลือดต่ำ อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญของเชื้ออื่นๆที่ศึกษากับระดับของโรคฟันผุทั้งในฟันแท้และฟันน้ำนม ทั้งสองกลุ่ม

ผลของการศึกษาลักษณะเคลือบฟันโดยวิธี polarized light microscopy, microradiography, และ scanning electron microscopy ในฟันน้ำนม 80 ซี่ โดย 23 ซี่มาจากกลุ่มที่มีระดับตะกั่วในเลือดสูง และ 57 ซี่ มาจากกลุ่มที่มีระดับตะกั่วในเลือดต่ำ ไม่พบลักษณะของเคลือบฟันที่ผิดปกติอันเนื่องมาจากตะกั่วในทั้งสองกลุ่ม

โดยสรุป ระดับของตะกั่วในเลือดสัมพันธ์กับความรุนแรงของโรคฟันผุที่เพิ่มขึ้น หลังจากควบคุมปัจจัยต่างๆที่สัมพันธ์การเกิดโรคฟันผุ แม้ว่าในการศึกษานี้ยังไม่สามารถอธิบายถึงกลไกของตะกั่วในการส่งเสริมความรุนแรงของโรคฟันผุ ว่าเกิดจากการมีผลต่อเชื้อในช่องปาก หรือ ส่งผลต่อปัจจัยทางด้านน้ำลาย หรือ ลักษณะของฟันที่เปลี่ยนแปลงไป ก็ตาม ผลของงานวิจัยฉบับนี้ก็แสดงให้เห็นว่าต้องมีมาตรการการควบคุมการกระจายของสารตะกั่วในพื้นที่ รวมถึงการคัดกรองเด็กกลุ่มเสี่ยงในพื้นที่ปนเปื้อนสารตะกั่ว นอกจากนี้ควรจัดให้มีมาตรการการป้องกันโรคฟันผุในเด็กกลุ่มนี้โดยเฉพาะ และให้ความรู้ในการดูแลสุขภาพช่องปากรวมถึงการติดตามดูแลอย่างต่อเนื่อง

คำหลัก : สารตะกั่ว, โรคฟันผุ, เด็ก, เชื้อแบคทีเรียในช่องปาก, ฟัน

Executive Summary

• Introduction and rationale

Among several heavy metal pollutants, lead has gained a special attention due to its significant content in leaded gasoline and paint, products that are easily spread in the nature. Furthermore, it virtually affects several organ systems and causes neurological, physiological, and behavioural problems in children. Its toxicity ranges from decreased intelligence quotient (IQ), decreased stature and growth, and impaired hearing acuity, at low blood lead concentrations, to encephalopathy, memory loss, and dealth at high blood lead concentrations. One of the most importance of lead is its accumulation in bone and teeth. This may subsequently have an impact on tooth development and dental caries occurence.

The accumulation of lead in teeth has raised the interest to whether children living in lead contaminated areas have increased susceptibility to dental caries. An association between environmental lead exposure and increased prevalence of caries has been reported. However, little is known about the strength of this association, the influence of other environmental factors, host factors as well as the etiological role of lead on the caries process and caries susceptibility.

In southern Thailand with highly prevalent caries, many areas have long been known to contaminate with lead from industry, such as ship reparing. The project aims at proving whether children living in lead-contaminated area have increased dental caries and how lead enhance the dental caries.

• Objectives

The overall aim of the study is to verify the influence of childhood lead exposure among 6-12 years old children on dental caries occurrence in contaminated rural area of southern Thailand.

Main specific objectives are

1. To test whether the following caries associating factors are different among children with low and high PbBs.

1.1 Stimulated whole saliva: salivary flow rate, salivary pH, buffer capacity,

salivary lead level

1.2 Microbilogical data: Mutans streptococci level, *Lactobacillus spp.* level,

other bacterial strains

2. To test the hypothesis that dental caries experience in deciduous teeth (dfs) and in permanent teeth (DMFS) are significantly higher in children with high levels of lead identified by tooth level and blood level of lead after adjustment for potential confounders.

Two subsidiary objectives were added:

Subsidiary objective 1: To evaluate the level of selected oral bacterial species in low lead-exposed children and high lead-exposed children in association with dental caries.

Subsidiary objective 2: To examine the morphology of enamel of primary teeth in children with high levels of lead in serum and compare with a group of children with low levels of lead in serum, by means of polarization microscopy (PLM) and scanning electron microscopy (SEM).

- **Research methodology**

1. Study design

The study was a school-based cross-sectional observational study.

2. Study population and sampling technique

Three primary schools established around shipyard area of Huakhao subdistrict, Singkanakhon District, Songkhla Province, southern Thailand were purposively selected to obtain a study subject residing around the shipyards. This area has been known to be endemic area for lead exposure by environmental lead contamination contributed by the use of Pb_3O_4 in ship-repair industry. Only two schools were selected (Banhuakhao, Watbosub School) by the reason for safety.

The inclusion criteria for study subjects were schoolchildren who aged between 6-10 years in two selected primary schools: Watbosub School and Banhuakhao School, and received a parental consent.

The children who had not been residing in the shipyard area at birth were excluded from the study. The children were also excluded if they had serious illness or health problem. In case of noncooperation, they were excluded from the study. At last, a total number of subjects for the project were 292: 101 from Watbosub School and 191 from Banhuakhao School.

3. Data collection process

1. Questionnaire interview

Interview was face-to-face, using a structured questionnaire. Interviews were divided into 2 parts: with their parents or caretakers and with the study subjects. The questionnaire for the caretaker covered major information regarding socio-economic status and information about the child. The interview with the study subjects covered the oral health behaviour and sugar-containing sweet intake.

2. Lead analysis of blood

Venipunctures were performed at schools by three licensed nurses from Singhanakhon District Hospital. Approximately 4 ml of venous blood specimen was taken from cubital vein. The blood lead level was determined by Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS) with Zeeman background correction (HITACHI Model Z-8200). The cut-point level of <10 µg/dl was presently considered within the range of acceptation (CDC 1991).

3. Clinical examination

All clinical examination was carried out in portable dental chair with natural light. Clinical oral examination started with oral hygiene determination, followed by dental caries examination.

3.1 Oral hygiene: The Oral Hygiene Index composed of the combined "Debris Index" and "Calculus Index" was used to assess oral hygiene status of the subjects.

3.2 Dental caries: Examination of caries status followed the criteria of the World Health Organization (1997). The examiner was blinded for children's blood lead level during the examination.

4. Saliva parameters

4.1 Saliva flow rate

Each child was asked to chew a paraffin sheet. The amount of stimulated whole saliva produced in five minutes was recorded to obtain the saliva flow rate.

4.2 Saliva pH and buffer capacity

Salivary pH and buffer capacity were measured immediately using pH indicator strips scaled from 3.8 to 8.1, with a resolution of 0.2-0.3, in accordance with the manufacturer's instructions (Macherey-Nagel, Germany)

5. Microbiological Investigation

5.1 Investigation of mutans streptococci and lactobacilli using Spatula method

Mutans streptococci and lactobacilli were determined using spatula method (Kohler and Bratthall 1979). The number of colonies after culture of plates was counted.

5.2 Collection of plaque samples by toothbrush method and analysed by checkerboard DNA-DNA hybridization technique

Plaque sample was collected by toothbrush method. The sample was transported to the laboratory at Faculty of Dentistry, Prince of Songkla University and stored at -20°C until used for analysis of other caries related bacteria by checkerboard DNA-DNA hybridization technique at the laboratory of Göteborg University, Göteborg, Sweden.

6. Tooth sample

6.1 Collection of tooth sample

The study subjects were informed since our first visit to keep their teeth shedding in the period, and to take it to their schoolteachers. The tooth specimens were collected and stored dry till they were prepared for the morphological examinations. The deciduous tooth was extracted if it meets the following criteria: prolong retention of deciduous tooth while permanent tooth already erupts in the mouth.

6.2 Examination of tooth morphology-mineralization

All teeth were macroscopically examined for caries, enamel aberrations and other findings. Afterwards, they were serially sectioned in bucco-lingual direction to a thickness of 100-120 μm. Central sections were used for the microscopic investigations.

6.3 Polarized light microscopy

All sections were examined in polarized light, both dry-in-air as well as after water imbibition for 24 hours, in an Olympus polarizing microscope. The PLM examination was carried out without knowledge of which group the specimen belonged to.

6.4 Scanning electron microscopy

Seven sections from the Pb-group and five from the control group were selected for the SEM analysis. The selection was based on the PLM examination and the sections represented the various morphological findings. The enamel surfaces of sections were investigated in a Philips SEM 515 at 15kV.

7. Collecting drinking water for the fluoride measurement

Drinking water samples from every main source in Moo 1-8 of Huakhao subdistrict were collected and assessed using a fluoride electrode (Thermo Orion, Model 710A plus, Sciencetech Co., Ltd.).

• Summary of the findings and Conclusion

The present study provides the caries status of the children living around shipyards, previously known as a lead-contaminated area (Maharachapong 2005). The results indicated that overall the prevalence of dental caries of children in the study area was extremely high (44% in permanent teeth and 94% in deciduous teeth). More importantly, children with high lead levels (PbB $\geq$10 μg/dl) significantly had more deciduous caries than those with low lead levels (PbB <10 μg/dl) The association has still been evident after confirmation with multivariate regression analysis. The difference was not observed in permanent teeth, possibly as a result of low number and duration of permanent teeth at risk. The finding from microbiological part of this study did not indicate that any oral bacterial species was associated with dfs, DMFS in high lead-exposed children, although two species, i.e. *L. acidophilus*, *V. parvula* were found different between high lead-exposed and low lead-exposed children. This study also failed to demonstrate the difference in enamel morphology between children with high PbB and those with low PbB.

In conclusion, blood lead exposure of children living around shipyards is associated with an elevated severity of dental caries in deciduous teeth, but not in permanent teeth. The mechanism could not be explained by its action through oral bacteria, saliva parameters, and enamel morphology in this study. Based on available evidence of recent in vitro study, lead could interfere with tooth formation, thus leading to alteration of tooth structure, possibly resulting in changing resistance of enamel crystals to caries attack during the caries process. Further investgation is most likely to be doing with the property on solubility of enamel with lead apatite.

Contents

Contents (Continued)

Contents (Continued)

Abbreviations and Symbols

CDC	Centers for Disease Control and Prevention (USA)
CI	Confidence interval
CFU	Colony forming unit
dfs	The number of decayed and filled deciduous surfaces
DMFS	The number of decayed, missing and filled permanent surfaces
GFAAS	Graphite furnace atomic absorption spectrophotometer
ml	Millilitre
MRG	Microradiography
PbB	Blood lead
PLM	Polarized light microscopy
ppm	Part per million
ppb	Part per billion
SD	Standard deviation
SEM	Scanning electron microscopy
WHO	World Health Organization
mg/kg	Milligram per kilogram
μg/dl	Microgram per decilitre

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

    1. Nattaporn Youravong, Virasakdi Chongsuvivatwong, Alan F. Geater, Gunnar Dahlen, Rawee Teanpaisan. Lead associated caries development in children living in a lead contaminated area, Thailand. The Science of the Total Environment, in press (Accepted 6 May 2005).

    2. Nattaporn Youravong, Virasakdi Chongsuvivatwong, Alan F. Geater, Gunnar Dahlen, Rawee Teanpaisan, Wolfram Dietz, Jorgen G. Noren. Morphology of enamel in primary teeth from children in Thailand exposed to environmental lead. The Science of the Total Environment 348, 73-81.

2. การนำผลงานวิจัยไปใช้ประโยชน์

    ผลงานวิจัยมีประโยชน์ในเชิงวิชาการ โดยนำไปใช้ในการพัฒนาการเรียนการสอน และเป็นการสร้างนักวิจัยใหม่

3. อื่นๆ : ได้นำเสนอผลงานในที่ประชุมวิชาการ ดังต่อไปนี้

    1. Lead and dental caries in children (Proposal). A collaborative meeting "Exchange information and identify potential collaborative research between Prince of Songkla University and Universiti Sains Malaysia". Universiti Sains Malaysia. March 5 –6, 2002. (Oral presentation)

    2. Dental caries and lead exposure in schoolchildren living a shipyard area (Preliminary results). Graduate Conference. Prince of Songkla University. March 12, 2003. (Oral presentation)

3. Dental caries and lead exposure in schoolchildren living a shipyard area (Preliminary results). Annual Research Conference. Epidemiology Unit, Prince of Songkla University. March 18, 2003. (Oral presentation)

4. Dental caries and lead exposure in children living in a shipyard area of southern Thailand. RGJ - Ph.D. Congress IV. Chonburi, Thailand. April 25-27, 2003. (Poster presentation)

5. Dental caries and lead exposure in children living in a shipyard area of southern Thailand. 81st International Association for Dental Research (IADR). Göteborg, Sweden. June 25-28, 2003. (Poster presentation)

6. Microbiology in toothbrush samples of children exposed to lead in southern Thailand. Annual Research Conference. Epidemiology Unit, Prince of Songkla University. February 28-March 1, 2005. (Oral presentation)

Chapter 1

INTRODUCTION

Among several heavy metal pollutants, lead has gained a special attention due to its significant content in leaded gasoline and paint, products that are easily spread in the nature. Furthermore, it virtually affects several organ systems and causes neurological, physiological, and behavioural problems in children. Its toxicity ranges from decreased intelligence quotient (IQ), decreased stature and growth, and impaired hearing acuity, at low blood lead concentrations, to encephalopathy, memory loss, and dealth at high blood lead concentrations. One of the most importance of lead is its accumulation in bone and teeth. This may subsequently have an impact on tooth development and dental caries occurence.

The accumulation of lead in teeth has raised the interest to whether children living in lead contaminated areas have increased susceptibility to dental caries. An association between environmental lead exposure and increased prevalence of caries has been reported. However, little is known about the strength of this association, the influence of other environmental factors, host factors as well as the etiological role of lead on the caries process and caries susceptibility.

In southern Thailand with highly prevalent caries, many areas have long been known to contaminate with lead from industry, such as ship reparing. The project aims at proving whether children living in lead-contaminated area have increased dental caries and how lead enhance the dental caries.

1. Introduction to southern Thailand and study area

Southern part of Thaland consists of fourteen provinces. Songkhla is one of five provinces bordering Malaysia. It overlooks Songkhla Lake on the West and the Gulf of Thailand on the East. Besides it connects the cultures of the East and the West, it has also been a boundary between the Buddhist and the Islamic cultures. Therefore, southerners had unique culture and language.

General information of the study site

The study area is in Haukhao subdistrict, Singhanakhon District, Songkhla Province. Singhanakorn District is located to the north of Songkhla, 28 kilometers from Maung District. Of eleven subdistricts, Huakhao is one subdistrict in Singhanakhon, where villages lie along the east coast. Huakhao subdistrict covers an area of approximately 15 m^2. It consists of 8 villages (village number 1-8) and 2,564 households. The size of total population is 14,448: 7,243 male and 7,205 female. Most are Muslim (73.5%), and the rest (26.5%) is Buddhist. People earn for their livings by the following occupation: shopkeeper (32.9 %), fishery (24.8 %), employee in factory (22.5 %), employee (17.2 %), government officer (1.4 %), and farmer (1.2 %). Three primary schools, Watbosub School, Banhuakhao School, and Bankhaodang

School are situated at village number 2, 6, 7 respectively. There are 2 shipyards located at village number 1 and 2.

This area is known to be an endemic area for lead exposure. Lead has been environmentally contaminated both in soil and dust of the area (Maharachpong 2005). Industry of shipyard involves the use of PB_3O_4 as a material for repairing, and distribution of lead in surrounding area.

2. The oral health status of children in Thailand

Dental caries is a demineralization process that results in tooth decay. The carious process affects all mineralized dental tissues of the tooth, namely enamel, dentine, and cementum, and is caused by the acid production of microorganisms using fermentable carbohydrates from the diet (van Houte 1994; Fejerskov 1997; Seow 1998). It can ultimately result in demineralization of the mineral portion of these tissues followed by disintegration of the organic material. Progression of the lesion into dentine can result in bacterial invasion and subsequently inflammation and necrosis of the dental pulp and spread of the infection into the periapical tissues.

Ministry of Public Health carried out national oral health surveys of six age groups of 3, 5-6, 12, 15, 35-44, and 60-74 – years old in 1977, 1984, 1989, 1994, and 2000-2001 respectively (Department of Health 1989; 1994; 2001). It was found that more than 70% of 6 years old children in Thailand were affected by dental caries of primary and permanent teeth as seen in Table 1.

The data on dental caries level indicated the increasing prevalence.

Table 1 Percentage of children affected by dental caries in the national oral health surveys.

Age group	1984	1989	1994	2000-2001
3 years old (primary teeth)	_	66.5	61.7	65.7
6 years old (primary teeth)	71.6	83.1	85.1	87.4
6 years old (mixed dentition)	74.4	82.8	85.3	87.5
12 years old (permanent teeth)	-	-	53.9	57.3

From 1984 to 2001 the prevalence of dental caries occurrence tended to increase in all age groups. The percentage of caries-free children was low. Table 2 shows that the southern region had the highest percentage of dental caries in all age groups, compared to other regions.

Table 2 Percentage of children affected by dental caries by region in 2000-2001.

Region	6 years old (primary teeth)	12 years old (permanent teeth)
Whole country	87.4	57.3
Central	87.9	58.8
North	85.7	58.2
North-eastern	89.0	50.3
South	91.4	64.8

The number of teeth affected by dental caries per person is described as DMFT or the number of decayed, missing (due to caries), and filled teeth in permanent dentition and as dmft or the number of decayed, missing (due to caries), and filled teeth in deciduous dentition. Both indicators tended to increase over years as seen in Table 3. Furthermore the number of carious teeth as well as the prevalence of caries was also the highest in the southern region for all ages (Table 4).

Table 3 Mean number of decayed, missing, and filled teeth in deciduous dentition (dmft) and in permanent dentition (DMFT) in the national oral health surveys.

Age group	1984	1989	1994	2000-2001
3 years old (dmft)	_	4.0	3.4	3.6
6 years old (dmft)	4.9	5.6	5.7	6.0
12 years old (DMFT)	1.5	1.5	1.6	1.6

Table 4 The number of DMFT and dmft by urbanization and region in 2000-2001.

Region	3 years old (dmft)	6 years old (dmft)	12 years old (DMFT)
Whole	3.6	6.0	1.6
Central	3.8	5.8	1.6
North	3.8	5.7	1.7
North-eastern	3.9	6.2	1.4
South	4.0	6.8	2.1

These findings give a conclusion that dental caries is a significant and widespread problem among Thai children, especially in the south. It should be noted that the 'D' and 'd' are usually the major components of the respective DMFT and dmft because most carious teeth are left untreated.

3. Literature review

3.1 Lead poisoning and general health impact

3.1.1 Sources of lead and variety of exposure routes

Lead is one of the most important and widely distributed pollutants in the environment in terms of global contamination and health impact (Goldman 1995; Jarup 2003; Billings et al. 2004). Metallic lead is a naturally occurring bluish-gray metal found in small amounts in the earth's crust. Lead compounds are used for many industrial processes, for example, tetraethyl lead and tetramethyl lead as gasoline additives, and PbO, Pb_3O_4, $Pb(SO_4)_2$ (Suwanabun 1975) for the production of batteries. Since the early 1920s, tetraethyl lead has been used in gasoline as an anti-knocking agent increasing octane rating and improving fuel efficiency. This produced large amounts of lead oxide in automobile exhaust. Considering the potential toxic effects associated with its exposure, approximately 50 nations renounced the use of lead in gasoline (Landrigan 2002). Thai government launched a national policy to campaign for the use of lead-free gasoline in Bangkok in 1991, and nationally in 1996. Currently, methyl tertiary butyl ether has been used as a substitute in unleaded gasoline. Other manufacturing activities involving the use of lead include the production of ammunition, some metal products (such as sheet lead, solder, pipe, brass/bronze foundries etc.), ceramic glazes, paint, medical equipment (such as radiation shield for protection against X-ray, surgical equipment, etc.) (Leighton et al. 2003; CDC 2005; Nuwayhid et al. 2003; Hardison et al. 2004; Perry et al. 2005). Rubber products and plastics industries, steel welding and cutting operations, and lead compound manufacturing industries also involve the use of lead. Some types of paints and pigments that are used in facial make-up or hair coloring contain lead (Rahbar et al. 2002). Lead-containing home remedies and cosmetics were used by some ethnic groups (Wolfe 2000).

Cigarette smoking is also an important factor contributing 0.96-2.00 μg of lead per one cigarette (Chiba and Masironi 1992). By those concentrations of lead in filter-tipped cigarettes, approximately 100 ng of lead per cigarette was released to contaminate the air (Suna et al. 1991). Thus passive smoking especially parental smoking also plays an important role in exposure of children to lead (Mannino et al. 2005; Chiba and Masironi 1992). Malara et al. (2004) reported the higher concentration of lead and cadmium in shed deciduous teeth from 6-13-year-old children who exposed to smoke in apartments, compared with those from children whose aparments were free of smoke. Thus, by the industries and human activities, lead has resulted in the surrounding environment being severely polluted. For example, the emission of lead into the atmosphere may come from lead containing paint or vehicle exhaust contribution (Okuda et al. 2004). After the leaded gasoline was banned in Bangkok in 1991, the average air lead level in Bangkok has continued to decrease (Ruangkanchanasetr and Suepiantham 2002).

The deposit of lead in the surrounding soils may be caused by mining waste, other industrial activities, or human activities, including atmospheric fallout of lead emission (Hardison et al. 2004; Martley et al. 2004; Madejon et al. 2002; Chojnacka et al. 2005; Vandecasteele et al. 2002). The extent of contamination depends on many factors such as size and duration of the industrial operation, atmospheric conditions, seasonal variation, physical and chemical properties of the emitted particles, and the amount of lead released to the atmosphere (Martley et al. 2004; Cenci and Martin 2004; Okuda et al. 2004). Lead emitted from industry in form of waste water can result in the lead contamination of water or water sediment (Cenci and Martin 2004; Andrews and Sutherland 2004). The study by Andrews and Sutherland (2004) revealed that road-deposited sediments (RDS) with high lead concentration from automobile usage were flushed into stream systems and contaminated stream bed sediments. The lead levels may be taken up by plants and animals from contaminated soils, as well as from contaminated water and polluted air (Chojnacka et al. 2005; Madejon et al. 2002; Meador et al. 2005; Liu et al. 2005). Thereby, in polluted area, the transfer of toxic element from soil to plant is of great concern. It was found that the edible leaves or stems of some crops were more heavily contaminated than seeds or fruits (Liu et al. 2005). Even after the remediation of contaminated soils, some plants growing in these remediated soils accumulated lead in their tissues (Madejon et al. 2002). Therefore, daily intake of foodstuffs can introduce lead to the body through ingestion (Santos et al. 2004).

If the daily intake of the amount of lead from the crops grown at the contaminated sites has exceeded the level of the

Recommended Dietary Allowance or Provisional Tolerable Daily Intake (RDA or PTDI) for adult (mg day^{-1}) set by WHO, it could have a hazard effect on human’s health.

The bioavailability of metal ions, e.g. Pb, Cd, Cr, As, etc. and its accumulation in plant is controlled by a variety of factors such as plant species and its physiological properties, type and property of soil, and the mobility of metal (Liu et al. 2005; Chojnacka et al. 2005). Therefore the presence of the metals in soil does not imply that they are available to plants.

Contamination of lead in air, dust, soil, drinking water and food crops harmfully elevated blood lead levels, especially in children. Exposure to lead and lead compounds occurs in several ways. Living near waste sites or environment with a heavy accumulation of lead may lead to a high level of lead exposure by breathing air, drinking water, eating foods, or swallowing or touching airborne dust (Albalak et al. 2003; Rahbar et al. 2002; Leighton et al. 2003; Reissman et al. 2002; Nowak 1995). Families of people working in the industrial processes may also be exposed to high lead level when those people bring home lead dust on their work clothes (Rahbar et al. 2002). Taking lead-containing foods or drinking water, contacting with deteriorating leaded paints, working in jobs where lead is used, using health-care product, doing hobbies that lead is used are the routes which lead can enter the bodies (Nuwayhid et al. 2003; Albalak et al. 2003). In addition, lead can enter the body through skin contact, e.g. contact with lead-containing dust and some cosmetics (such as some types of lip balm), hair colorants and lead acetate-containing dye. Lead passing through the skin contributes very little lead to the body. Most of lead particulates (diameter greater than 5 micrometers) enter the body through swallowing. Accidental ingestion may occur from skin contamination while eating, drinking, smoking, or applying cosmetics.

The lead poisoning can be found not only in the group of industrial workers, but also in the susceptible groups to the exposure, such as infants, children, and pregnant women. The lead can be transplacentally acquired during the intrauterine life if the mother is exposed (Clark 1977). Pregnant women with heavy lead exposure may deliver prematurely, and their babies are more apt to have low birth weight (Andrews et al. 1994; Bellinger 2005). There was a significant correlation (correlation coefficient=0.77) between blood lead concentrations of maternal venous blood and infant cord blood in a lead-affected community (Clark 1977).

Children are more susceptible to the harmful effects of lead or lead poisoning than adults. Even at low levels of exposure, lead can affect their mental and physical growth. Activities, such as playing on the ground (Maharachpong 2005), the contamination of dust or soil lead from hand-to-mouth activities make them easier to take lead (Rahbar et al. 2002). Swallowing of lead in house dust or soil is also an important exposure pathway for children.

3.1.2 Health effects of lead

Most of its chemical forms of lead are toxic to multiple organ systems (such as the liver, kidneys, lungs, brain, spleen, muscles, and heart) and produce adverse effects that are well-documented (Needleman 1991). Its effects are the same whether it is breathed or swallowed. Although lead deposition may occur in any tissue, the main target of lead toxicity is the nervous system both in adults and children. It is especially harmful to the developing brains of fetuses and young children. Lead interferes with neuronal migration, cell proliferation and synapse formation (Needleman 1991). The consequences are loss of intelligence and disruption of behaviour (Needleman and Gatsonis 1990; Schwartz 1994; McMichael et al. 1994). An increase in blood lead from 10 to 20 μg/dl can decrease IQ by 2.6 points (Schwartz 1994). Children experienced various symptoms, ranging from constipation and retardation to encephalopathy (Goldman 1995). At extremely high levels of lead (70 μg/dl or higher), lead can cause coma, convulsions, and even death. Even low levels of lead are harmful and are associated with decreased stature and growth, and impaired hearing acuity. Other problems of childhood lead poisoning also include learning abilities, bahavioural problems, and mental retardation.

Centers for Disease Control and Prevention, USA. (CDC 1991) considers children to have an elevated level of lead if the amount of lead in the blood is above or equal to 10 μg/dl (level of CDC concern). When the blood lead level rises, the severity of the outcome increases, as summarized in Table 5. Medical treatment is needed in children with the blood lead concentration higher than 45 μg/dl.

Table 5 The health effects of lead by the childhood blood lead level

The effect on health	blood lead level (μg/dl)
Death	130-140
Encephalopathy	90
Nephropathy	80
Frank anemia	70
Colic	60
Hemoglobin synthesis	20-40
Nerve conduction velocity	10-20
Vitamin D metabolism	10-15
Developmental toxicity (IQ,hearing,growth)	10

Source: CDC, 1991 (http://www.cdc.gov/)

The blood lead levels as low as 10 μg/dl are associated with harmful effects on children's learning and bahaviour. It has been therefore suggested by CDC to prevent the occurrence of blood lead levels of 10 μg/dl and above in children. Among the health effects of lead listed by CDC, dental health has not been included, probably due to inadequacy of past studies.

3.2 The situation of lead exposure in Thai children

Legislation of unleaded oil in Thailand

Lead poisoning in children has been of concern. Worldwide countries have been realizing and providing some measures to protect them. Legislation of unleaded gasoline is an example of measure enforced in many countries (Landrigan 2002). The high content of lead in gasoline is serious issue due to its combustion emitted to the environment. International Conference on Environmental Threats to the Health of Children, held in Bangkok in March 2002, brought 35 countries and organizations from the Southeast-Asian and Western-Pacific regions to increase awareness on environmental health hazards (Suk et al. 2003). The Bangkok statement, a pledge resulting from the conference proceedings, indicated the declaration to reduce or eliminate exposure to toxic elements such as lead, arsenic etc. to prevent children from these toxic elements (Suk et al. 2003). An example was to promote legislation for removal of lead from all gasoline, paints, water pipes and ceramics. Thai government launched a national policy to campaign for the usage of unleaded gasoline since January 1, 1996 nationally.

3.2.1 Blood lead level of Bangkok children before and after ban for leaded gasoline

Before legislation

In 1993-1996, the study on the blood lead level in Bangkok children aged 6-11 years who visited Ramathibodi Hospital showed that it was 10.32 ± 4.74 μg/dl (range 0.57-28.4 μg/dl) and the proportion of children having the blood lead level greater than 10 μg/dl was 51.1% (Ruangkanchanasetr et al. 1999). Another study was concurrently conducted during 1994-1996 in 5-6 year-old children residing in Bangkok showed that the average blood lead level was 8.3 ± 3.1 μg/dl (range 3.1-24.3 μg/dl) and 75 out of 330 children had blood lead greater than 10 μg/dl (Muangnoicharoen et al. 1998). A high proportion (22.7-51.1%) of children with blood lead level ≥10 μg/dl before ban for leaded gasoline were indicated.

After the reduction of lead levels due to unleaded gasoline usage ever since 1996, childhood blood lead level in Bangkok tended to decrease. Vichit-vadakarn et al. (2002) found that the mean blood lead level of Bangkok schoolchildren from 6 primary schools in 2000 was 5.6 μg/dl, as compared to 8.6 μg/dl in 1993. Only 2.7% had the blood lead level greater than 10 μg/dl, whereas, in 1993 the percentage of children exceeding 10 μg/dl was about 26%. The decline of blood lead may be explained by the reduction of the average ambient lead level in Bangkok from 2.33 $\mu g/m^3$ in 1992 to 0.79 $\mu g/m^3$ in 1995 (Office of Environmental Policy and Planning 1995; 1998). The unleaded gasoline policy is an effective measure in lowering the lead in the environment leading to the improvement of children's blood lead levels.

However, the condition mentioned is still unsatisfactory, as the problem of cognitive development constitutes a great proportion of children whose blood lead levels were higher than those of acceptation. This is possibly due to other remaining sources of lead. Thus, other sources of lead should also be of great concern. Lead in paint is another source of lead poisoning in Thailand. As in the findings by Vichit-vadakarn et al. (2002), another important risk factor of exposing lead was house paint. Children, whose family member employed as painter, had a risk of having their blood lead levels exceeding 10 μg/dl nine times higher than those who did not have family member as painter. Consequently, any measure to control lead-based paint or any other source of lead has to be enforced as the ban for leaded gasoline.

3.2.2 Lead contamination in water at Clitty Creek, Kanchanaburi Province.

A depression storm during September-October 1997 caused flooding in Kanchanaburi Province. As a result, a tailing pond in a nearby ore refinery plant was flooded and lead-contaminated water spilled into the Clitty Creek. The release of lead pollutants into the Clity Creek has been contributed to the contamination of environment and lower Clity villagers for many years. Subsequently, the water lead level of the Clitty Creek in 1998 was found 0.39-0.75 mg/l which was 40-75 times higher than those of acceptance (WHO standard level in drinking water is 0.01 mg/l)(Poopinyokul 2001). High lead levels were also found in soil deposit around the plant (5,800-102,574 mg/kg) and aquatic life in the Lower Clitty Creek (5.45-81.79 mg/kg dry weight)(Tantanasrikul et al. 2002).

Many organizations have been of awareness of the lead contamination in the area. Department of Health, Ministry of Public Health reported the blood lead levels of the villagers in 1999 and indicated the existing problem of lead poisoning in the Clitty Creek as shown in Table 6 (Poopinyokul 2001).

Table 6 The blood lead levels of villagers in the Clitty Creek

Age	The blood lead level (μg/dl)			
	February(1999)	No.	March(2000)	No.
0-6 years old	23.56(13.56-36.04)	39	26.45(12.56-48.80)	37
7-15 years old	28.30(21.08-33.05)	8	27.04(17.13-35.09)	11
16years old and more	26.31(8.58-41.36)	72	29.35(17.55-48.60)	54

Source: http://www.anamai.moph.go.th/factsheet 2001

This table indicates the extensive problem of lead poisoning in the Clitty Creek villagers and faliure to control the problem in the area in one-year period. There should be additional intervention after that.

Another study under the co-operation of Ministry of Health, Minstry of Science, Minisrty of Industry, and Kanchanaburi office of Public Health revealed that 68 children, aged under 15 years and followed yearly during 1997-1999, had high blood lead levels seen in Table 7)(Tantanasrikul et al. 2002). Simultaneously, environmental remediation was begun. Methods included shutting down the refinery, preventing contaminated soils from entering into populated areas, creating a lead-free water source, and providing an alternative source of protein and avoiding consumption of animal meat from the creek.

Table 7 Average blood lead level from 1997-1999.

Year	Condition	n	Average PbB (μg/dl)
1997	Baseline level at initial survey	68	27.75 ± 5.4
1998	First survey after environmental deleading	68	30.64 ± 4.5
1999	Second survey	68	30.30 ± 5.1

Source: Tantanasrikul et al. 2002

From the table, the environmental remediation was considered a priority to mode of treatment. However, only the environmental deleading was insufficient to solve the problem of lead poisoning. After two-year follow up period, the children who had blood lead level greater than or equal to 25 μg/dl were observed. Those with persistently high blood lead levels (18 children) received medical intervention in the form of chelation therapy. Overall average of blood lead before and after chelation were 30.30 ± 5.10 μg/dl and 18.73 ± 7.50 μg/dl respectively. Therefore, the chelation therapy may be a mode of treatment when environmental contaminated decreased.

3.2.3 Lead contamination in Pattani and Yala Province.

The high lead content in Pattani River and sediments were reported since 1986 (Varatorn 1998). Pattani River flows past Yala Province, Pattani Province to the Gulf of Thailand at Pattani Bay. Activities, which are major sources of Pattani River contamination, are many tin-mining areas and manufacturing around the mouth of Pattani River including ship-reparing industry (Varatorn 1998). Although the mine of Bannang Star District and Yaha District abandoned for many decades due to falling price of tin, the remainder of lead existed in the surrounding environment. Additionally, there have also been other operating mines in Yaring District, Pattani Province and Tepa District, Songkhla Province.

An evidence during the period Feb-Mar 1995 revealed a high proportion of 6-15 year-old schoolchildren with blood lead level ≥ 10 μg/dl, in mining areas and at the mouth of Pattani River (Geater et al. 2000). The blood lead levels of schoolchildren in Tambol Thamthalu, Bannang Sta District and Tambol Tachi, Yaha District of Yala Province; Tambol Sabarang, Muang District of Pattani Province, were high, as seen in Table 8 (Geater et al. 2000). These regions were reported to have high environmental lead content. Tambol Thamthalu was a rural region containing several tin mines abandoned approximately 7 years prior to the study and Tambol Tachi formerly contained a tin mine permanently closed about 20 years prior to the study. Tambol Sabarang is situated at the mouth of Pattani River, where high lead contamination of river sediments was reported. No mining activity has been conducted in this region.

Table 8 Blood lead concentration by school

School	No. of children	Blood lead (μg/dl)		No.of children	No. of children
		Geometric Mean	Geometric SD	≥10 μg/dl (%)	≥20 μg/dl (%)
Ban Thamthalu (Bannang sta)	46	15.91	1.30	44 (96)	11 (24)
Ban Tangkadeng (Bannang sta)	127	12.39	1.36	94 (74)	9 (7)
Ban Tachi (Yaha)	105	7.96	1.31	23 (22)	0 (0)
Tesabal 3 (Muang)	61	14.76	1.23	60 (98)	6 (10)
Ban Sabarang (Muang)	57	10.92	1.28	37 (65)	0 (0)

Source: Geater et al. 2000

The table showed the high magnitude of problem affecting children residing in the lead-contaminated area. Consequently there should be more additional effective measures for environmental remediation and/or medical intervention in the area.

The findings reported by Song-ug-sorn (2002) showed a marked decrease of lead contamination in children of Ban Tangkadeng in 2002. One hundred and sixty-nine schoolchildren aged 5-15 years from Ban Tangkadeng School, Tambol Thamthalu, had blood lead between 0.9 and 51.32 μg/dl with mean of 6.89 μg/dl and SD of 4.79 μg/dl. Twenty among them (16%) had blood lead level ≥ 10 μg/dl. However, in these high risk areas, the government should have implemented some measures systematically both in environmental deleading and surveillance. High blood lead children should be followed and screened for medical intervention.

A shipyard in Pattani Province is one of the risk areas of lead contamination due to a direct contact to lead pollutants in manufacturing. Findings of four shipyards in the Pattani Province, revealed elevated blood lead levels of shipyard workers and their children residing in the area as seen in Table 9-10 (Provincial Health Authority of Pattani 2000).

Table 9 Number of workers and blood lead levels in four shipyards in Pattani

Shipyard No.	The distribution of blood lead level (μg/dl)				
	<25	25-<40	40-60	>60	Total
1.	12	15	17	11	55
2.	3	6	6	2	17
3.	2		1	1	4
4.	1	6	2	3	12
Total	18	27	26	17	88

Source: Provincial Helath Authority of Pattani 2000.

Table 10 Distribution of blood lead levels of shipyard workers'children in Pattani Province

The blood lead level(μg/dl)	Number of children	
	First	Second
<10	3	1
10-<15	3	6
15-<20	9	3
20-<45	12	6
45-<70	2	1
Total	29	17

Source: Provincial Helath Authority of Pattani 2000.

Claulking workers in shipyard industries in Pattani Province were at high risk for exposing lead. Approximately 50% had blood lead level exceeding 40 μg/dl that needed to be remedied. The children in their families also faced the risk. Almost all (26/29) had blood lead level exceeding the acceptable level (10 μg/dl). After four months of education and behaviour modification, their second blood lead values decreased. However some had still had high blood leads, they should be further followed. The most important point is how they behave to prevent themselves from re-contamination of lead, and what the government should implement to lower the occupational risks.

3.2.4 Lead contamination in Songkhla Province.

Risk at exposing lead in children around shipyard industry does not confine to Pattani Province. Songkhla Province, coastal area, also consists of this industry. Findings of a study (Maharachpong 2005) revealed elevated blood lead levels of children residing around shipyards in Singhanakhon District, as shown in Table 11.

Table 11 Distribution of blood lead levels of children around shipyards in Songkhla Province

The blood lead level(μg/dl)	Number of children
<10	151
10-<15	106
15-<20	33
20-<25	18
>25	12
Total	320

Source: Maharachpong 2005.

Most Children, residing around shipyards anywhere, contaminated lead into the body in the level of unacceptation.

3.3 Linkage between tooth decay and childhood lead exposure

Deposition of lead is much greater in skeletal tissues than in soft tissue (Steenhout 1982), since Pb, similar to Sr and F, are bone-seeking elements. Exposure over a period of years is indicated by Pb levels in bones and teeth (Blanusa et al. 1990; Bercovitz and Laufer 1991; Hac et al. 1997; Selpes et al. 1997). Therefore, dental tissues are considered reliable biomarkers for determination of exposure to lead (Omar et al. 2001; Grandjean et al. 1984; Fergusson and Kinzett 1989). The lead concentrations in whole tooth and in the constituent matrices: dentine and enamel have been used as a means of assessing exposure to lead, and relationship to human health in a number of studies (Delves et al. 1982; Karakaya et al. 1996; Selpes et al. 1997; Purchase and Fergusson 1986; Grobler et al. 2000; Fosse et al. 1995; McMichael et al. 1994). Besides contributing to the assessment of lead exposure in children, many epidemiologists focus their interests on whether children living in contaminated area have increased susceptibility to dental caries. Environmental excess of Pb has been shown to be associated with increased incidence of caries (Barmes 1969; Barmes et al. 1970; Anderson et al. 1976; Anderson et al. 1979). Therefore it has been hypothesized that the exposure to lead is likely to be related to more caries. Whether lead accumulation in the tooth increases susceptiblility to caries remains to be investigated.

Conclusively, tooth lead is of particular interest in two main ways: reflecting the body burden of lead and possibility of increasing the dental caries.

3.3.1 The studies of association between lead exposure and dental caries

The role of trace elements in the development of tooth decay has been an area of study since the identification of protective effects of fluorides. The advantage effect of fluoride was first reported in 1909 in Colorado, USA, and led to the fluoridation of some public water supplies in several countries (Levy 2003; CDC 2001; Petersen and Lennon 2004). Inevitably, the recognition that water-containing fluorides was protective against caries led to investigations of the possibility that other trace elements also might affect dental health. Therefore, other trace elements in food and water have been linked with dental caries later. Dental epidemiologists have provided some convincing evidence that trace elements can affect the health of community population. Molybdenum was reported to be associated with reduced caries prevalence whereas selenium and lead might have adverse effects (Davies and Anderson 1987). Curzon and Crocker (1978) reported the positive association between tooth decay and Mn, Cu, and Cd, and the negative association with F, Al, Fe, Se and Sr. However the negative finding of Pb should be intepreted with high caution due to small sample size (n=47, from 451 teeth) and the possibility that one element may confound the effect of another.

Results of animal studies showed an association between lead exposure and caries. In a recent study, pregnant rats were randomized to receive either lead-contaminated water or lead free-water; the exposure was continued until the rat pups were weaned. The mean smooth surface and sulcal surface caries scores were higher among the lead exposed rat pups than the non-exposed rat pups (Watson et al. 1997). Human epidemiological studies also report an association between lead exposure and dental caries. Direct association between soil lead and increased caries was reported in Papua-New Guinea (Barmes 1969; Barmes et al. 1970). Likewise, Anderson et al. (1976) investigated a possible lead effect by examining dental caries prevalence in 12-year-old children resident in Tamar valley in the west of England. The Tamar valley is an old mining area characterized by widespread heavy metal contamination. The results revealed an apparent small reduction in caries associated with mineralised area with the exception of one part, Bere Peninsula, where children had a raised caries level. The Bere Peninsula was the principal producer and refiner of lead ore in the nineteenth century and all the agricultural soils are contaminated by lead. A similar study in Somerset (southwest England), where soils were polluted with lead and cadmium, showed no statistical difference between the dental health of 12- and 15-year-old children in the affected area compared with neighbouring uncontaminated areas (Anderson et al. 1979).

Brudevold et al. (1977) reported a positive association between caries and lead level in teeth using enamel biopsy. The group of high enamel lead had the highest caries scores, both in terms of DFT and DFS. Gil et al. (1996) reported that tooth lead concentrations increased with caries (DMFT). Tooth samples from caries-free subjects exhibited a significantly lower lead content than the other groups. It positively correlated with presence of dental plaque, *Streptococcus mutans* and *Lactobacillus salivalis* counts, dental abrasion, tooth colour, and toothbrusing frequency. The pH of saliva was significantly negative-correlated with caries. However, being a cross-sectional study this association could hardly claim causality.

Moss et al. (1999) in a secondary data analysis of the third National Health and Nutrition Examination Survey (NHANES III, reported a significant association between blood lead exposure and caries on permanent teeth of children aged 5-17 years with odds ratios of 1.36-1.66 for caries in permanent teeth. Although they appear to be low, such odd ratios imply a sizable burden of disease: an additional 2.7 million children in USA may have caries as a result of lead exposure.

The association study did not always end up with positive association result. Campbell et al. (2000) linked the records of children in the caries database, examined through a program conducted by the Eastman Dental Center, to a database of blood lead levels maintained by the local country health department. The results demonstrated that lead exposure >10 μg/dl at toddler was marginally associated with deciduous caries among school-age children (OR=1.77; 95%CI, 0.97-3.24; p-value=0.07). For permanent caries (DMFS), the odds ratio was 0.95 (95%CI, 0.43-2.09; p=0.89). This study is limited by its low statistical power (<30%) for detecting low odds ratio of caries in permanent teeth (n=248). However, there were some limitations that might have caused an underestimation of the association. First, the subjects were examined at a young age (mean = 8.4 years), where the caries of the permanent teeth was low (the mean DMFS score was only 0.5). Therefore, sufficient time may not have elapsed for caries to become manifested on permanent teeth. Second, the examination was school-based caries screening program to establish cavity lesions for treatment. This will underestimate the prevalence of caries.

The recently published study by Gemmel at al. in 2002 assessed the association between blood lead level and dental caries in children 6-10 years old screened for enrollment in the Children's Amalgam Trial, a study designed to asess potential health effects of mercury in silver fillings. Positive association was reported in urban group, but not in rural children. The difference between the association in urban and in rural settings might reflect the presence of residual confounding in the former setting.

Based on available evidences, it can be presumed that the exposure to lead is likely to influence the susceptibility to dental caries.

3.3.2 Possible mechanisms of lead as a risk factor for caries

For caries to develop, the three main factors, i.e. cariogenic bacteria, fermantable carbohydrates, tooth and environment, have to be interacting. The question is how exposure to lead can enhance susceptibility to dental caries. The mechanism of action of lead is not completely established. There are possible mechanisms which lead could enhance caries.

3.3.2.1 Saliva flow rate

Presence of lead may affect adversely the function of the salivary glands. Lead may directly act on gland tissue to inhibit saliva formation. A decrease in salivary flow rate impairs the buffering function of saliva in protecting the tooth against bacterial acids and leads to an increase in the prevalence of caries. One of the most likely mechanisms by which lead may interfere with saliva formation is its interaction with Ca^{2+} metabolism. In an animal study by Watson et al. in 1997 provided evidence when rat pups born to mothers who were exposed to lead in drinking water had 40% more cavities than pups born to unexposed mothers. This may, in part, be because pups born to exposed mothers produced 30% less saliva than those born to unexposed mothers. Another evidence by Craan et al. in 1984 showed that administration of lead in rats significantly diminished stimulated salivary flow rates. The phenomenon has not been examined in the human.

3.3.2.2 Interaction with fluoride

A possible explanation may be that lead complexes with fluoride, and renders fluoride insoluble and unable to exert its cariostatic effects, and thus diminish its protective effect on enamel demineralization. Although the study by Tabchoury et al. (1999) concluded that lead did not interfere with the protective effect of fluoride in rats, it is necessary to study the interaction of these elements in tooth.

3.3.2.3 Lead absorbed enamel increases caries susceptibility

3.3.2.3.1 Pre-eruptive and post-eruptive lead exposure to susceptibility to dental caries

Pre-natal and peri-natal lead exposure may predispose children to dental caries. The effect of Pb at the pre-eruptive vs. post-eruptive stage on subsequent caries is not clear. Grobler et al. (2000) and Purchase and Fergusson (1986) reported higher lead concentration in dentine than in enamel and the highest lead concentration was in the area of circumpulpal dentine. The finding from Grobler's study gave a figure of 16% increase in lead concentration of dentine compared with whole tooth (Grobler et al. 2000). A decline in lead away from the pulp cavity (Purchase and Fergusson 1986) to the bulk of enamel, and the equivalent levels of lead content in individual match-paired impacted and erupted third molars as researched by Bercovitz and Laufer (1992), suggested that Pb enters the tooth from the blood stream. The results from Bercovitz's study confirmed that the lead accumulated before the eruption of tooth, presumably during dentine formation, and without any contribution from the oral environment. However, the distribution of lead concentration in the enamel was proposed to vary with different area. Higher lead content was found in surface enamel than in the enamel-dentine junction, implying that part of the lead accumulation in enamel may be acquired at the post-eruptive period from the oral environment (Brudevold et al. 1977; Purchase and Fergusson 1986). In Brudevold's study, it was also revealed the level of lead as high as 4,000 ppm. in the outer layer of enamel where lead declined from the surface to the enamel-dentine junction. It was thus suggested that the lead accumulation in enamel might relate to the contact of the teeth with saliva and plaque lead in the mouth. This particularly affected not only the surface lead level surface, but also the concentration of lead in different parts of the enamel. The result from Schamschula et al.’s study (1977) demonstrated that the levels of lead appeared to be concentrated in dental plaque from 2.7 to 54.7 ppm. Therefore, the incorporation of lead into the tooth structure may be implicated in a reduced resistance of enamel to dental caries. Gerlach et al. (2002) showed that regions of maturing enamel were not as hard in mice exposed to lead as in control animals.

In conclusion, the effect of lead in promoting caries is likely to be the combination of the effect of pre- and post-eruptive lead exposure.

3.3.2.3.2 Lead absorbed enamel may result in defective enamel

A possible mechanism is that lead incorporating into enamel may result in defective enamel that is more susceptible for caries. It was suggested that Pb might first adsorb to hydroxyapatite crystal surfaces and later took up positions within the structure (Curzon and Cutress 1983). Some of the properties of Pb hydroxyapatite produced under laboratory conditions were established (Bhatnagar 1970). Yet the explanation that lead in enamel/dentine may give a higher susceptibility surface has still been elucidated.

The recently published study by Gerlach et al. in 2002 demonstrated that mineralization of enamel was altered in the incisors of rats exposed to lead in the drinking water, in downwards trend. Even though the more decreased mineral content of enamel in rats exposed to lead than that in control group did not reach significance at the confidence level of 95%, this observation was be of importance in view of the plausible role of lead on enamel formation. Additionally, the finding on microhardness of the enamel formed during exposure to lead indicated that regions of maturing enamel were significantly not as hard in mice expose to lead as in control animals.

In conclusion, three different mechanisms were explored concerning lead exposure and dental caries: inhibiting saliva formation, affecting enamel formation, and interfering with fluoride in saliva.

So far, there has not been any study of role of lead on the interaction with caries inducing microorganisms. If this of interaction is known, prevention and control of caries in lead contamination area can be planned more properly.

4. Rationale

Data from previous epidemiological studies and animal-based research support the concept that lead may be a caries-promoting element. In area where the prevalence of dental caries increases, a high lead contamination is reported. Nevertheless the association between lead exposure and dental caries has still been ambiguous and the mechanism is not well established. As the occurrence of dental caries is affected by multiple factors, careful control for possible confounding is needed. Few studies were carried out to examine the association between lead exposure and dental caries in the human population to check the consistency with causal relationship shown in the laboratory animal studies. The notion that lead toxicity is an important risk factor for caries, if true, would have important implications to tackle caries problem. If the association is spurious, resources can be reallocated more efficiently.

Thus, there is a need to carry out a series of research project to obtain answer for those questions.

5. General objective

The overall aim of the study is to verify the influence of childhood lead exposure among 6-12 years old children on dental caries occurrence in contaminated rural area of southern Thailand.

6. Specific objectives

Main specific objectives are

1. To test whether the following caries associating factors are different among children with low and high PbBs.

    1.1 Stimulated whole saliva

        1.1.1 Salivary flow rate

        1.1.2 Salivary pH

        1.1.3 Buffer capacity

        1.1.4 Salivary lead level

1.2 Microbilogical data

1.2.1 Mutans streptococci level

1.2.2 *Lactobacillus spp.* level

1.2.3 Other bacterial strains

2. To test the hypothesis that dental caries experience in deciduous teeth (dfs) and in permanent teeth (DMFS) are significantly higher in children with high levels of lead identified by tooth level and blood level of lead after adjustment for potential confounders.

We add two subsidiary objectives:

Subsidiary objective 1:

To evaluate the level of selected oral bacterial species in low lead-exposed children and high lead-exposed children in association with dental caries.

Subsidiary objective 2:

To examine the morphology of enamel of primary teeth in children with high levels of lead in serum and compare with a group of children with low levels of lead in serum, by means of polarization microscopy (PLM) and scanning electron microscopy (SEM).

7. Conceptual framework

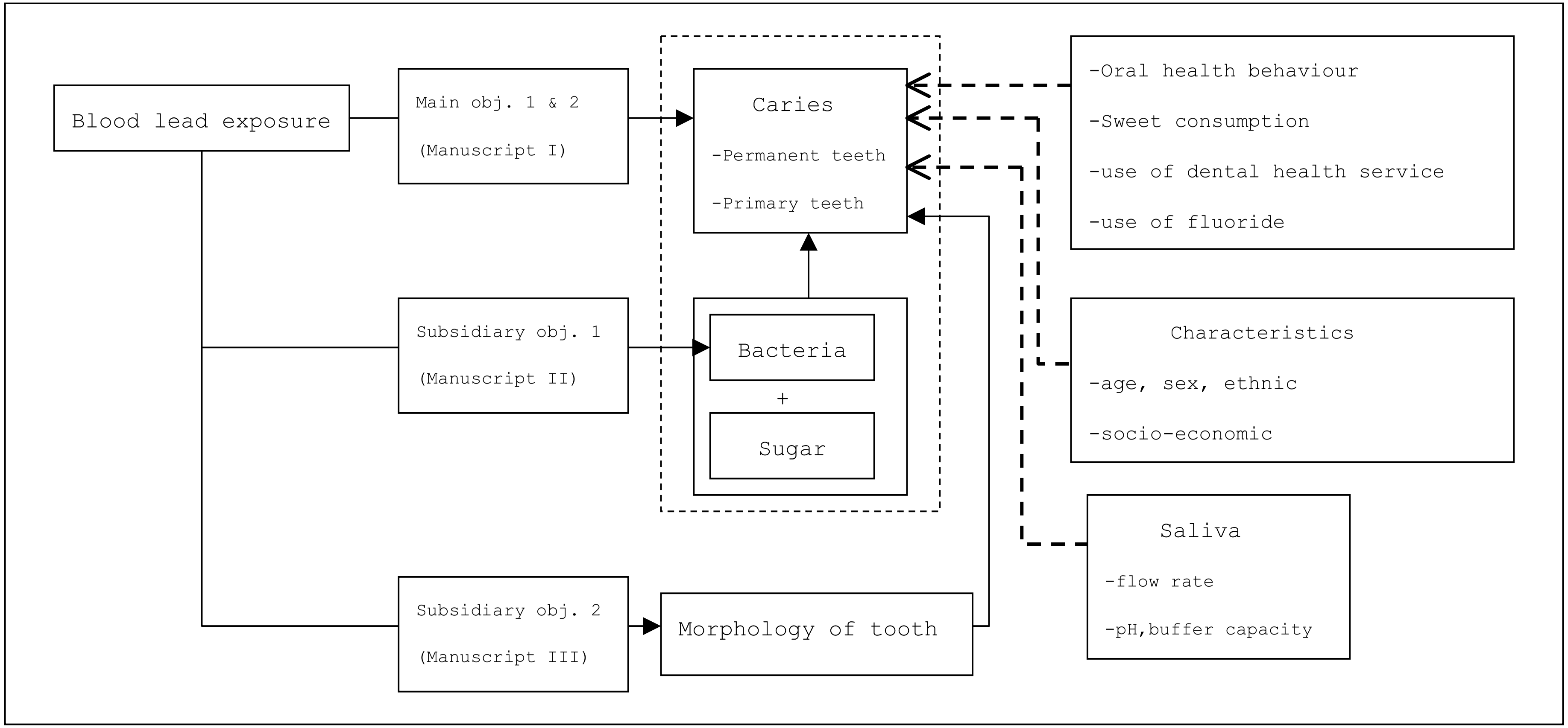


Figure 1 Diagram of conceptual framework and objectives & corresponding manuscripts

Chapter 2

MATERIALS AND METHODS

This chapter describes the methodology of the studies for all parts. The methodologies in this chapter are also addressed in Chapters 3 where the manuscripts of studies were included. Nevertheless the materials and methods in each of those manuscripts were shortly briefed in necessary points. The methodologies in this chapter give details completely and demonstrate all procedure of the thesis work.

1. Study design

A school-based cross-sectional observational study was carried out. The children in two schools where located around the shipyard area were enlisted, the children aged 6-10 years were enrolled to the study to document dental caries problem related to lead exposure.

2. Study population and sampling technique

Target population: 1,054 6-10-year-old schoolchildren from 3 primary schools around shipyards in Huakhao subdistrict

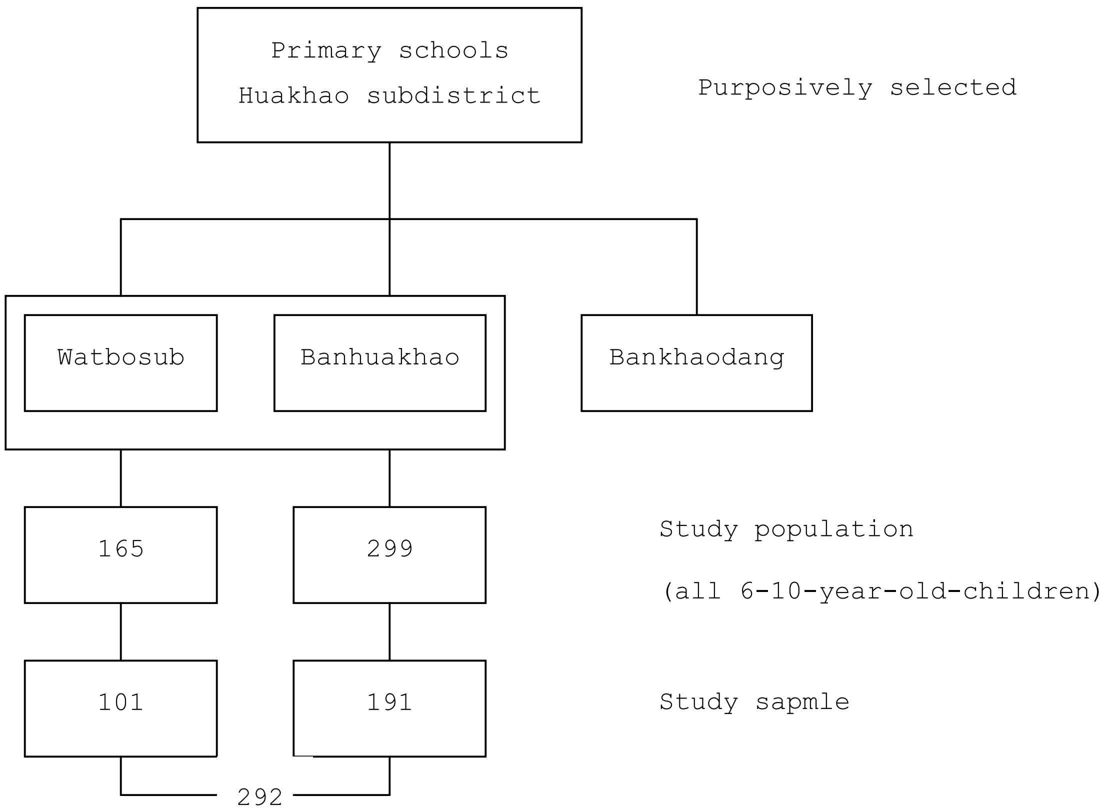
Study population: 464 6-10-year-old schoolchildren from 2 selected primary schools

Three primary schools established around shipyard area of Huakhao subdistrict, Singkanakhon District, Songkhla Province, southern Thailand were purposively selected to obtain a study subject residing around the shipyards. This area has been known to be endemic area for lead exposure by environmental lead contamination contributed by the use of Pb_3O_4 in ship-repair industry (Maharachpong 2005). One school (Bankhaodang School) was dropped and only two schools were selected (Banhuakhao, Watbosub School) by the reason for safety.

The study recruited 6-10-year-old children whose teeth were mixed dentition (comprising both primary and permanent teeth). The inclusion criteria for study subjects were schoolchildren who aged between 6-10 years in two selected primary schools: Watbosub School and Banhuakhao School, and received a parental consent.

From the list of schoolchildren of two schools, all children aged 6-10 years, 165 for Watbosub and 299 for Banhuakhao School were included and asked to bring the consent form informing about the aim of study and all procedure to their parents/caretakers. After the consent form returned with approved signature from the parent/caretaker, 144 out of 165 was permitted for Watbosub School, and 223 out of 299 for Banhuakhao School. Then an appointment for the oral examination and interview of caretaker was determined. The children who had not been residing in the shipyard area at birth were excluded from the study. The children were also excluded if they had serious illness or health problem. Fifty-five children were excluded due to uncontinuous residence in the area. Because of many clinical procedures involved in this study, participated children had to be cooperative in our visits. In case of noncooperation, they were excluded from the study. During the process of taking blood sample, twenty children could not be taken blood sample due to their noncooperation. At last, a total number of subjects for the project were 292: 101 from Watbosub School and 191 from Banhuakhao School.

Figure 2 Sampling frame and study sample



Study variables

Dependent (outcome) variables

1.Decayed, missing, and filled teeth and surface in deciduous dentition (dmft, dmfs)

2.Decayed, missing, and filled teeth and surface in permanent dentition (DMFT, DMFS)

Independent (explanatory) variables

1. Blood lead level (μg/dl)

Acidogenic bacterial count(*Lactobacilli* spp., mutans streptococci)

Salivary parameters

1. Salivary flow rate
2. Salivary pH
3. Buffer capacity

Socio-economic factors

1. Gender
2. Religion
3. The average income of family a year

Oral health behaviours

1. Self-care practice
2. Sugar intake (dietary consumption)

Others

1. Oral hygiene
2. The number of tooth and tooth surface present

3. Sample size calculation

The total sample size required was 300 schoolchildren, approximately 150 schoolchildren in each group of lead exposure. Actual sample size in the study was 292 schoolchildren completing clinical examination, venous blood sample, and interview.

The sample size was first calculated based on our pilot study for the estimation of variance (σ^2) and the minimal worthwhile benefit (d or μ_1-μ_2) of caries experience, and based on the other previous study (Maharachpong 2005) for the estimation of the ratio of high lead exposure to low lead exposure (r).

The principal concern for statistical power in the study was to investigate the association between blood lead exposure and caries experience level. Based on testing hypothesis of different exposures of lead on the dental caries, sample size calculation was performed using the equation illustrated below.

$$\boxed{N1= [(1+1/r)(Z_{\alpha/2}+ Z_{\beta})^2]\ \sigma^2 / d^2}$$

N_1 = number of subjects in group with lower level of lead

N_2 = number of subjects in group with higher level of lead

$r = N_2/N_1 = 1$

$\alpha = 0.05$, $Z_{\alpha/2} = 1.95996$

$\beta = 0.8$, $Z_{\beta} = 0.8416$

$\sigma^2 / d^2 = 3:1$

$N_1 = [\ 2(1.95996+0.84156)^2\ (4.5)^2\ ]/\ (1.5)^2\ =\ 141.27$

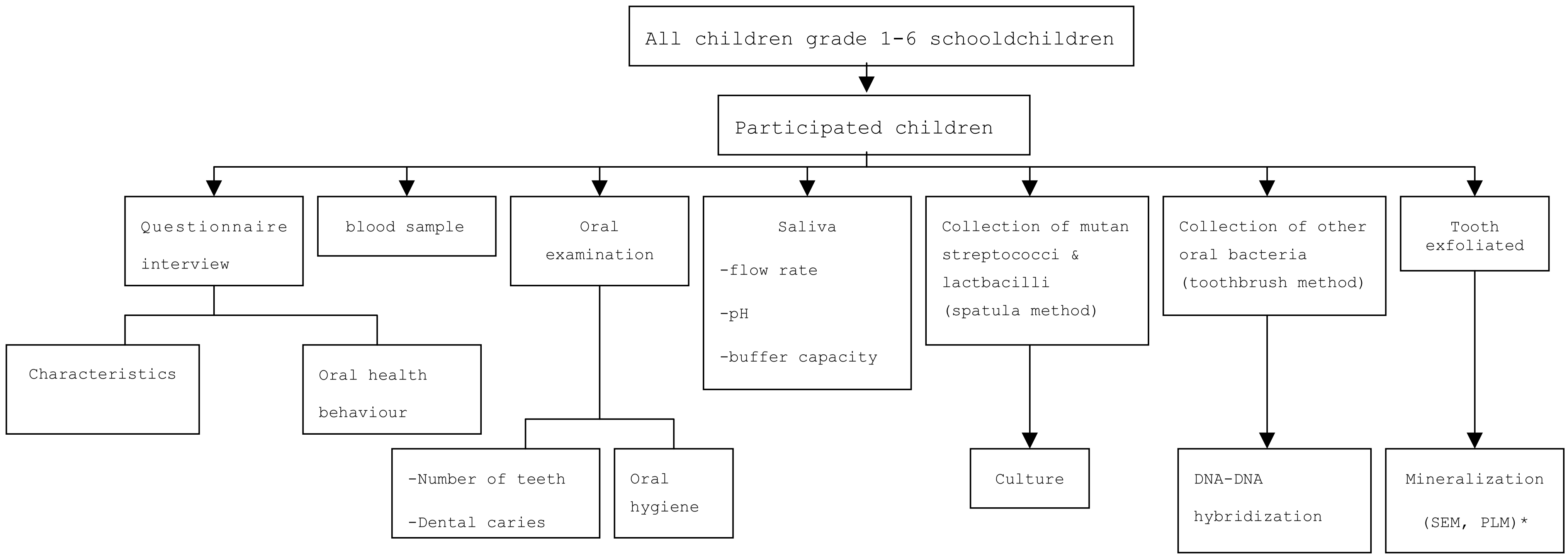
With a probability of type I error (α) of 0.05 and a power of 0.80, the calculated sample size was 284 schoolchildren for the project (142 for each group of lead exposure). This sample size was used for testing hypothesis in the main objective of the study. However, for the subsidiary objectives where the laboratories were intensive, the sample size was reduced. Therefore the total number of children required was approximately 300 to overcome all uncertainty.

4. Ethical considerations

This study used data from venous blood sample. The subjects and their parents were all asked for informed consent. The detailed project together with a consent form was approved by the Ethical Committee, Faculty of Medicine, Prince of Songkla University, Hatyai, Thailand prior to the start of the project (Appendix C and D).

5. Data collection process

Figure 3: Sequence of data collection process



* SEM= scanning electron microscopy, PLM= polarized light microscopy

5.1 Questionnaire interview

Interview was face-to-face, focusing on the children and their families regarding to personal data, socio-economic status, dietary habits, and oral health behaviour, using a structured questionnaire. Six interviewers and six recorders were trained and supervised on the technique of interview and objective of each item of question by the author of thesis. They were undergraduate students, recruited based on the criteria of ability to speak southern dialect well. Interviews were divided into 2 parts: with their parents or caretakers and with the study subjects.

5.1.1 Interview with parents

As study samples are children, their main caretakers were interviewed to obtain the reliable information about the child. The interview was conducted either at school or at home, depending on their convenience, mostly at home.

The questionnaire for the caretaker covered major information regarding these respects:

1.Personnel information about the child: sex, age, religion, location (village), level of education, general health, and serious illness.

2.General information: number of years of residing in the area, occupation, workplace, and educational level of their parents, estimated family income in a year.

5.1.2 Interview with children (subjects)

The interview with the study subjects was carried out at school. The part of questionnaire designed for them covered the following topics:

1. Oral health behaviour: frequency of tooth brushing, the use of toothpaste containing fluoride and frequency, other methods in tooth cleaning and (toothpicks, floss, chewsticks), frequency of dental visit

2. Sugar containing sweet intake: the kind and frequency of sugar consumption (food and drink)

5.2 Lead analysis of blood

Collection of blood sample

Venipunctures were performed at two study schools by three licensed nurses from Singhanakhon District Hospital. Approximately 4 ml of venous blood specimen was taken from cubital vein using a disposable syringe and needle. The specimen was kepted in heparin-coated lead-free plastic bottle with a sealed screw cap, in an icebox during transportation and stored at 4° C until analyzed for lead level in a few days later. The blood lead level was determined by Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS) with Zeeman background correction (HITACHI Model Z-8200). The three levels of reference whole blood were set for constructing a standard curve for each batch of assay. The method gives the detection limit as low as 1 ppb or $0.02x10^{-9}$ g, and provides the measurement with coefficient of variation less than

3%. The cut-point level of <10 μg/dl was presently considered within the range of acceptation (CDC 1991).

5.3 Clinical examination

Clinical oral examinations were performed by only one examiner throughout the study period, with a chair-side dental assistant who was also responsible for recording the clinical data, and another assistant who held the name list of all study subjects and flowed them for the clinical examination in each day, by blinding the examiner about the name of subjects and their blood lead levels.

Examination area

The area for conducting examination was the primary school. All clinical examination was carried out in portable dental chair.

Examination posture

The children were examined in supine position. The chair faced the opening through which the light enters.

The sequence of clinical oral examination started with examination of oral hygiene status and dental caries status.

1.5.3.1 Oral hygiene

The Oral Hygiene Index composed of the combined "Debris Index" and "Calculus Index" was used to assess oral hygiene (Greene and Vermillion 1960). The Oral Hygiene Index was performed on the facial surfaces of 4 teeth (tooth no. 16,11,26, and 31), and the lingual surfaces of 2 teeth (tooth no.36 and 46). Dental explorer and plain mouth mirror were used for the examination.

The scores and criteria for oral debris are:

0: no debris or extrinsic stain present

1: soft debris covering not more than one third of the tooth surface, or the presence of extrinsic stains without any debris regardless of surface area covered

2: soft debris covering more than one third, but not more than two thirds of the exposed tooth surface

3: soft debris covering more than two thirds of the exposed tooth surface

The scores and criteria for oral calculus are:

0: no calculus present

1: supragingival calculus covering not more than one third of the exposed tooth surface

2: supragingival calculus covering more than one third, but not more than two thirds of the exposed tooth surface

3: supraginggival calculus covering more than two thirds of the exposed tooth surface

1.5.3.2 Dental caries

Oral clinical examination started with oral hygiene determination using Oral Hygiene Index as above, followed by dental caries examination. Examination of caries status followed the criteria of the World Health Organization (1997) using a plain mouth mirror and a probe (see Appendix A). The examiner was blinded for children's blood lead level during the examination. The examiner adopted a systematic approach to the examination of dental caries, proceeding in an orderly manner from one tooth or tooth space to the adjacent tooth or tooth space. It was started from upper right second molar (tooth no.17) to upper left second molar (tooth no.27) in upper dentition, and from lower left second molar (tooth no.37) to lower right second molar (tooth no.47) in lower dentition. The sequence of the tooth surfaces examined in each tooth was occlusal surface, mesial surface, buccal surface, distal surface, and lingual surface respectively.

5.4 Saliva parameters

5.4.1 Saliva flow rate

Saliva was collected immediately after the completion of the oral health status examination of each child. Each child was asked to chew a paraffin sheet similar to chewing bubble gum for 1 min to soften the paraffin, the saliva produced during this time was swallowed. Chewing was continued for a fixed time (5 min) with the same bolus of paraffin. The child was asked to expectorate the stimulated whole saliva by dribbling into 50 ml volumetric tube every 2 minutes until a fixed time had elapsed. Stimulated flow rate of saliva was calculated. The total amount of saliva produced was divided by 5 minutes to obtain the ml/min of flow rate.

5.4.2 Saliva pH and buffer capacity

Afterwards salivary pH and buffer capacity were measured immediately using pH indicator strips scaled from 3.8 to 8.1, with a resolution of 0.2-0.3, in accordance with the manufacturer's instructions (Macherey-Nagel, Germany) by wetting the pH paper and comparing immediately the color of the broader square in the middle with the one that had the same color. For buffer capacity, 1 ml of saliva was mixed with 3 ml of 0.005 M HCL in the plastic tube. Final pH was measured with pH indicator strip again as for salivary pH after leaving for 10 min. To eliminate carbon dioxide, shake the sample and remove the stopper. Buffer capacity and pH were classified into 2 groups: low (<6.5) and high (>6.5)

5.5 Microbiological investgation

5.5.1 Investigation of mutans streptococci and lactobacilli using Spatula method

To determine the caries associated bacteria (mutans streptococci and lactobacilli) in lead-exposed subjects compared to non-exposed subjects. Mutans streptococci and lactobacilli were determined using spatula method (Kohler and Bratthall 1979). A 1.8-mm.-wide wooden spatula was placed against the dorsum of the tongue until the spatula was visibly moistured. Any excess of saliva was removed by withdrawal of the spatula between closed lips. Each side of the spatula was then pressed against a disposable contact petri dish (Nunc, Denmark) containing Mitis Salivarius Bacitracin for mutans streptococci enumeration and Rogosa SL agar for lactobacilli. The plates were kept in a candle jar and transported to the laboratory within 3 hours after the specimen had been collected. The plates was then incubated at 37°C in the candle jar for 48 hours (Kohler and Bratthall 1979). The number of colonies on a 1.5 cm^2-predetermined area of the tip for each side pressed against the agar was microscopically counted for mutans streptococci, and visually for lactobacilli. They are scored on 0, 1-10 colonies, 11-50 colonies, 51-100 colonies, and more than 100 colonies. Mutans streptococci count was recategorized into 2 groups: <100 and >100 colonies corresponding to $<10^6$, $>10^6$ CFU/ml in saliva (Kohler and Bratthall 1979). While lactobacilli count was recategorized as <10, >10 colonies corresponding to $<10^4$, $>10^4$ CFU/ml in saliva. (Pongpaisan et al. 2000)

Remark: The classification of score 0,1-10, 11-99, and ≥ 100 corresponded to $0-10^3$, 10^3-10^4, 10^4-10^5, $>10^5$ respectively for lactobacilli. For mutans streptococci, the amount of <100 colonies obtained with spatula method corresponded to $<10^6$ CFU/ml in saliva.

5.5.2 Collection of plaque samples by toothbrush method and checkerboard DNA-DNA hybridization technique

Okada et al. (2000) introduced and recommended this procedure to be simple, and easily applied technique in children. Toothbrush method is a good alternative of collecting dental plaque from teeth represented a spectrum of oral species in the oral cavity in children and providing convenience and cooperation as performed in young children (Okada et al. 2000). To determine other oral bacterial species that may involve in dental caries such as *S.oralis, S.intermedia, S.sanguis etc.*, plaque sample was collected by toothbrush method.

A new toothbrush was available for each child. General research assistant accounted for brushing children's teeth. After that the plaque was removed from toothbrush into a 50 ml tube containing distilled water by vibrating the toothbrush several times until it was clean. Plaque sample was transported to the laboratory at Faculty of Dentistry, Prince of Songkla University and stored at -20°C until used for analysis of caries related bacteria by checkerboard DNA-DNA hybridization technique at the laboratory of Göteborg University, Göteborg, Sweden.

A. Bacteria tested

They included *Streptococcus mutans* (ATCC 25175), *Streptococcus sanguis* (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).

B. Checkerboard DNA-DNA hybridization technique

The Checkerboard DNA-DNA hybridization technique has been recently developed for detecting the panel of oral microbiota. The method allows for simultaneous detection of multiple species in the large number of samples generated in epidemiological studies against a number of different standard probes (Socransky et al. 1994; Papapanou et al. 1997a). In addition the technique is simple and does not depend upon the capability of bacterial growth, compared to culture method (Papapanou et al. 1997b). Thus the utilization of this technique is considered suitable for the detection of periodontal pathogen relating to dental diseases, particularly when the detection limit is not needed at very low level of bacteria. The assay does not require bacterial viability and is applicable in epidemiological research.

DNA-DNA hybridization method was performed as follows:

C. Processing of the bacterial plaque sample

Digoxigenin-labeled, whole genomic DNA probes were prepared using a labeling kit to examine occurrence of different microbial species. Genomic DNA was extracted from 18 microbial species. The samples were boiled for 5 min, neutralized, transferred on nylon membranes by means of blotting device, and immobilized by UV light and incubation at 120°C. After 2 hours of prehybridization, the DNA probes were allowed to hybridize overnight with the sample DNA using the second blotting device at 42°C. After a series of 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 substrate.

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 of species. The obtained chemiluminescent units were coded on a scale from 0-5, where 0 indicates no signal; 1, a signal density weaker than that of the low standard (i.e, < 10^5 bacteria); 2 signal density equal to that of the low standard (=10^5 bacteria); 3, signal density higher than that of the low standard but lower than that of the high standard (>10^5 but <10^6 bacteria); 4, signal density equal to that of the high standard (=10^6 bacteria); 5, signal density higher that the one of the high standard (>10^6 bacteria).

5.6 Tooth sample

5.6.1 Collection of tooth sample

The study subjects were informed since our first visit to keep their teeth shedding in the period, and to take it to their schoolteachers. The schoolteachers were asked to keep the naturally exfoliating deciduous teeth of study subjects in any situation leading to shedding of teeth, and to label the name of the child on a container in dry condition. The tooth specimens were collected and stored dry till they were prepared for the morphological examinations. The team periodically visited the schools to take all kept teeth every 2 weeks in 2 study schools throughout the data collection period of 4-6 months. The deciduous tooth was extracted if it meets the following criteria: prolong retention of deciduous tooth while permanent tooth already erupts in the mouth.

5.6.2 Examination of tooth morphology-mineralization

Prior to the histological and chemical investigations, all teeth were macroscopically examined for caries, enamel aberrations and other findings. Teeth were cleaned in distilled water. Afterwards, they were oriented and embedded in an epoxy resin (Epofix®, Electron Microscopy Sciences, Fort Washington, USA) and serially sectioned in bucco-lingual direction to a thickness of 100-120 μm with an Exakt diamond band saw (Exakt®, Exakt Vertriebs GMBH, Norderstedt, Germany). Central sections were used for the microscopic investigations.

1.1.3 Polarized light microscopy

All sections were examined in polarized light, both dry-in-air as well as after water imbibition for 24 hours, in an Olympus polarizing microscope employing strainfree objectives. The PLM examination was carried out without knowledge of which group the specimen belonged to.

1.1.4 Scanning electron microscopy

Seven sections from the Pb-group and five from the control group were selected for the SEM analysis. The selection was based on the PLM examination and the sections represented the various morphological findings. The sections were etched with 30 % phosphoric acid, carefully rinsed with distilled water, mounted on sample holders for the microscope, sputtered with gold, and investigated in a Philips SEM 515 at 15kV. Two intact non-etched teeth from each group were also coated with gold to reduce surface charging, by vapour deposition, and the enamel surface was examined in scanning electron microscopy (SEM).

1.7 Fluoride levels in drinking water

Fluoride is an important protective factor for dental caries. Besides fluoride toothpaste or fluoride supplement, fluoride levels in drinking water from various primary sources in the community was collected and assessed using a fluoride electrode (Thermo Orion, Model 710A plus, Sciencetech Co., Ltd.).

Collecting drinking water for the fluoride measurement

Drinking water samples from every main source in Moo 1-8 of Huakhao subdistrict were collected and labeled for the date of collection, location and source. In case of the same source of drinking water among villages, e.g. same well, the water sample from that source was once collected. If any village consumed more than one sources of water, the sample from each was collected (one sample/source).

6. Pilot survey

Before the collection of data had been started, the methods were tested. All procedure was performed using the same method in actual data collection following to these purposes:

1.To examine the validity and reliability of questionnaire, to calibrate and train interviewers.

2.Trial run of clinical dental procedure, blood sample including microbiological and laboratory examination.

3.To be intimately acquainted with key individuals and organizations in the community of study in order to enhance co-operation.

The intra-examiner consistency of dental examination in the pilot study was illustrated in Table 12 in the next topic.

7. Control of validity and reliability of measurements

7.1 Validity of questionnaire

The final draft of the questionnaire was discussed with three experienced researchers in the field of community dentistry: Dr. Wirat Eungpoonsawat, Dr. Angkana Thearmontree, and Dr. Jaranya Hunsrisakhun prior to testing in the field. All interviewers and recorders were trained in conducting the interview and recording the data in the fieldwork meticulously. Trial of questionnaire took place in village No.7 where was one of the study area. Fourteen interviewees in the same age of the study subjects were included in the trial. Afterwards, the questionnaire was modified before its actual conduction in the fieldwork in a few days.

During the study period of interviewing, all questionnaires conducted were verified by the main researcher at the end of each day. The ambiguous or incomplete records were repeated by the same interviewer and recorder in the next day.

The majority of people living in the study area could communicate with national Thai language, but some used southern dialect. Therefore the interviewers spoke with southern dialect to some extent. Thereby in this area where most residents were Muslim, we did not encounter any problem of communicating with Malayu language.

7.2 Reliability of clinical examination

Intra-examiner (the author of this thesis) test of reliability was performed to control the reliability of the clinical data twice, at pilot survey (before the study period) and during the period of study. Each used different group of children. Variables assessed were dental caries score.

- Before the study period

Intra-examiner reproducibility was performed on 10 randomly selected schoolchildren (1,260 tooth surfaces) of the study school to assess the consistency of the examiner on the dental caries one week apart.

- During the study period

The study period of clinical oral examination took place between November 2002 and January 2003. Thirty schoolchildren (10%) of the study subjects (4,680 tooth surfaces) were repeatedly examined for dental caries during the clinical period.

Table 12 and Table 13 show the results of calibration on the dental caries score records. The percentage level of agreement and kappa statistic was used to measure the degree of reliability. The percentage level of agreement and the kappa statistic were 89%, 0.87 respectively for the calibration prior the study period, and 97%, 0.97 respectively for that during the study period. These values indicated very good degree of agreement as illustrated in Table 12-13 (See Appendix A for code explanation).

Table 12 The intra-examiner agreement on dental caries scores prior to the study period

First Exam.	Second examination								
	0	1	4	8	9	10	11	14	Total
0	**403**	8	0	10	4	0	0	0	425
1	5	**15**	0	0	0	0	0	0	20
4	0	0	**0**	5	0	0	0	0	5
8	5	0	5	**186**	8	0	0	15	224
9	0	0	0	0	**15**	0	0	0	15
10	0	0	0	0	0	**355**	30	2	387
11	0	0	0	0	0	25	**141**	3	169
14	0	0	0	0	0	2	3	**10**	15
Total	413	23	5	201	27	382	179	30	**1,260**

See Appendix A for the code of dental caries. The code values from 0-19, the code omitted from the table gives the value of zero between the first and the second examinations.

Observed proportion of agreement (Po)

= (exactly observed agreement)/N

= (403+15+186+15+355+141+10)/(1,260) = 0.89

Percentage level of agreement = Po(100) = 89 %

Expected proportions of chance agreement (Pe) = 1/J = 1/8 = 0.125

$$\text{Kappa statistic} = \frac{(Po)-(Pe)}{1-(Pe)} = 0.87$$

Table 13 The intra-examiner agreement on dental caries scores during the study period

First	Second examination														
Exam.	0	1	2	3	4	6	8	9	10	11	12	13	14	19	Total
0	**1,944**	13	0	0	0	2	19	3	0	0	0	0	0	0	1,981
1	5	**434**	1	0	0	0	0	0	0	0	0	0	0	0	440
2	0	0	**0**	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	**4**	0	0	0	0	0	0	0	0	0	0	4
4	0	0	0	0	**5**	0	0	0	0	0	0	0	0	0	5
6	1	2	0	0	0	**39**	0	0	0	0	0	0	0	0	42
8	5	0	0	0	0	0	**756**	0	0	0	0	0	0	0	761
9	0	0	0	0	0	0	0	**0**	0	0	0	0	0	0	0
10	4	0	0	0	0	0	0	0	**912**	27	1	0	0	1	945
11	0	1	0	0	0	0	0	0	13	**413**	2	0	5	0	434
12	0	0	0	0	0	0	0	0	0	1	**19**	0	0	0	21
13	0	0	0	0	0	0	0	0	0	0	2	**0**	0	0	2
14	0	0	0	0	0	0	0	0	0	5	0	0	**40**	0	45
19	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	0
Total	1,959	450	1	4	5	41	775	3	926	446	24	0	45	1	**4,680**

See Appendix A for the code of dental caries. The code values from 0-19, the code omitted from the table gives the value of zero between the first and the second examinations.

Po = (1,944+434+4+5+39+756+912+413+19+40)/(4,680) = 0.97

Percentage level of agreement = 97%, Pe = 1/14 = 0.07

Kappa statistic = (0.97-0.07)/(1-0.07) = 0.97

8. Statistical analysis

All data from clinical, laboratory examination and interviews were explored by "check box" form to individually check availability of every part of records (see Appendix F). Thereafter, double data entry was performed into EpiData 2.0 ("The EpiData Association", Odense, Denmark) to validate an accuracy of data entry. Each part of data was separately filled out to easily manage when there was erroneous in the data entry, and merged together before the analysis. Afterwards the data was transferred by Stat Transfer6 to analyze in Stata statistical program version 7.0. Data exploration and cleaning was done to gain the integrity of the data, to check and correct mistakes in the data set.

The analysis was initially carried out using descriptive statistics, in form of frequency for categorical variable and mean for continuous variable. For analytical purposes, bivariate analysis was performed to explore the association between two parameters. Non-parametric statistics was used to compare the magnitude of dental diseases between lead exposed and non-exposed group. Chi-squared test was required for comparing oral health behaviour between lead exposed and non-exposed group. The associations between continuous scale of lead level and DMFS/dfs were performed using Spearman rank correlation coefficient.

The association may be plausibly confounded by other factors, multivariate regression analysis (multiple logistic regression model) was used to examine the association. The distribution of blood lead level is rather skewed, we normalized it into log base

2 of blood lead level and examine DMFS and dfs in each group of blood lead level. In addition to very high prevalence of dental caries in deciduous teeth (95%), that implies that almost every mouth could be seen at least 1 surface of deciduous caries. Therefore the prediction of dfs>5 surfaces was considered.

Chapter 3

Results

This chapter presents the results from all parts of the thesis, given in form of manuscript 1-3, serving each specific objective of the thesis (see Figure 1: conceptual framework). Manuscripts were written in different styles following to the instruction of each journal.

Manuscript 1 aimed to test the association between PbB and dental caries in deciduous and permanent teeth with adjustment for these factors.

Manuscript 2 had the objective to evaluate the level of 18 selected oral bacterial species in low lead-exposed children and high lead-exposed children in association with dental caries.

Manuscript 3 aimed to examine the morphology of enamel of primary teeth in children with high levels of lead in serum and compare with a group of children with low levels of lead in serum.

.

1. MANUSCRIPT 1

Lead associated caries development in children living in a lead contaminated area, Thailand

Nattaporn Youravong[1,*], Virasakdi Chongsuvivatwong[1], Alan F. Geater[1], Gunnar Dahlén[2], Rawee Teanpaisan[3]

[1] Epidemiology Unit, Faculty of Medicine

Prince of Songkla University, Hatyai, Songkhla, Thailand 90110

[2]Department of Oral Microbiology

Sahlgrenska Academy at Göteborg University, Göteborg, Sweden box 450 SE 405 30

[3]Department of Stomatology

Faculty of Dentistry, Prince of Songkla University, Songkhla,

Thailand 90110

Accepted: 6 May 2005 Published online: 20 June 2005, The Science of the Total Environment

Abstract

In an observational cross-sectional design, a sample of 292 children aged 6-11 years from two primary schools around a shipyard area, known to be an area contaminated with lead (from the industry), were examined to verify the cariogenicity of lead. The number of decayed and filled surfaces on deciduous teeth (dfs), and the number of decayed, missing, and filled surfaces on permanent teeth (DMFS), the salivary flow rate, pH, buffer capacity, oral hygiene, *Lactobacillus* spp. and mutans streptococci counts were recorded. The mean (range) of DMFS and dfs were respectively 1.3 (range 0-17) and 13.2 (range 0-45); and the geometric mean blood lead level (PbB) and SD were 7.2 and 1.5 μ g/dl. The level of dfs, but not DMFS correlated with the blood lead level (Rs=0.25, p=0.00/ Rs=0.09, p=0.14). The odds ratio for DMFS≥1 and dfs>5 for a doubling of PbB after adjusting for other factors were 1.28 (95%CI, 0.81-2.04; p value=0.35) and 2.39 (95%CI, 1.36-4.20; p value=0.004) respectively. The cariogenicity of lead is evident in deciduous teeth but not in permanent teeth for this age group.

Key words: dental caries, blood lead, salivary flow rate, salivary pH, buffer capacity, lactobacilli, mutans streptococci

Introduction

Lead, a heavy metal pollutant, is known to deposit in the central nervous system, causing developmental problems and calcified tissue including bone and teeth (Steenhout 1982; Grobler et al. 1991; Schwartz 1994; Goldman 1995). Primary (deciduous) teeth have been used as an indicator for lead exposure in a number of studies because they reflect the body burden of lead accumulation in chronic exposure (Karakaya et al. 1996; Selypes et al. 1997; Tvinnereim et al. 1997; Bloch et al. 1998).

Dental caries is a demineralization process affecting all mineralized tissue of the tooth and is caused by a complex process involving diet, bacteria and host factors. Certain cariogenic bacteria (for example; mutans streptococci or *Lactobacillus* spp.) are as important as salivary factors (such as salivary pH, buffer capacity, flow rate, and composition). In Thailand, more than 60% of 6-year-old children in all regions were affected by dental caries (National Oral Health Survey 2000-2001). In southern Thailand, the prevalence (93%) has been the highest ever since 1994.

Some human epidemiological studies have reported an association between lead exposure and prevalence of caries (Anderson et al. 1976, 1979; Davies and Anderson 1987). Brudevold et al. (1977) found that a group of children with high enamel lead content had higher caries scores than children with low lead enamel content. Gil et al. (1996) demonstrated that tooth lead concentration was an independent risk factor for caries. Moss et al. (1999) in the Third National Health and Nutrition Examination Survey (NHANES III) reported a significant association between blood lead exposure and caries prevalence on permanent teeth in children aged 5-17 years. Campbell et al. (2000) demonstrated a marginal association between dfs and blood lead measure and a significant association between dfs of lingual surfaces and blood lead measure among 248 school age children whose blood lead exposure was determined between 18 and 37 months of age. In the recent study, Gemmel et al. (2002) reported that the blood lead level was associated with the number of caries in primary teeth among urban, but not rural 6-10 years old children. These findings are consistent with animal studies. Rat pups whose mothers received lead-contaminated water during pregnancy showed higher caries scores than those whose mothers received lead-free water (Watson et al. 1997).

In the aforementioned studies by Gemmel et al. (2002) and Campbell et al. (2000), the analyses had adjustment for potentially confounding factors, such as socioeconomic status, ethnicity, and oral health behaviours. However, other important dental health factors such as the density of *S.mutans* and *Lactobacilli* spp., salivary flow rate, pH, and buffer capacity were not included. The objectives of our study were to test the association between PbB and dental caries in deciduous and permanent teeth with adjustment for these factors.

Materials and Methods

The Study population was a southern Thailand community, in Singhanakhon subdistrict of Songkhla Province, where ship repairing is a major industry. In this region all fishing ships are made of wood and need annual maintenance. In this process Pb_3O_4 powder is used without adequate personal and environmental protection. It has been shown in a similar type of community nearby that this industry led to the contamination of the environment and an increase of Pb in children (Geater et al. 2000).

Study population: Two primary schools: Watbosub School and Banhuakhao School, established near the shipyard area were recruited for our study. All children aged 6-10 years were enlisted from these two schools, and the children whose parents signed a consent form were invited to participate in the study. Then venous blood was taken from those children for PbB analysis. Children with major health problems were excluded. The project and the consent form were approved by the Ethical Committee, Faculty of Medicine, Prince of Songkla University.

Interview: The children's caretaker was interviewed at home by a trained interviewer using a structured questionnaire. Face-to-face data collected included personal data, socio-economic status, dietary habits, and oral health behaviour.

Blood lead (PbB): Venipuncture was performed at school by nurses from Singhanakhon District Hospital. A volume of 4 ml of blood from the forearm cubital vein was taken using a lead-free disposable syringe and needle, then transferred to a lead-free tube, kept in an ice-box during transportation and stored at 4°C before analysis of the lead level at the Faculty of Tropical Medicine, Mahidol University, Bangkok. The blood lead level was determined by Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS) with Zeeman background correction (HITACHI Model Z-8200). The three levels of reference whole blood were set for constructing a standard curve for each batch of assay. The method gives the detection limit as low as 1 ppb or 0.02×10^{-9} g, and provides the measurement with coefficient of variation less than 3%.

Oral hygiene: The Oral Hygiene Index (Greene and Vermillion,1960) was assessed by inspection of facial surfaces of tooth number 16,11,26, 31, and the lingual surfaces of tooth number 36 and 46. It consisted of the debris index and calculus index. The plaque index was defined as mild, moderate or heavy, and the calculus index as present or absent.

Dental caries: Every tooth was examined using a plain mouth mirror and a probe. Dental caries was defined by the number of decayed and filled surfaces in deciduous teeth and by the number of decayed, missing, and filled surfaces in permanent teeth according to the criteria of the World Health Organization (1997). All examinations were carried out by only one dentist with an intra-examiner kappa statistic of 0.96. The examiner was blinded for children’s blood lead level during the examination.

Salivary parameters: 15 ml of paraffin stimulated-saliva was collected after 5 minutes chewing. The saliva pH and buffer capacity were measured immediately using pH indicator strips scaled from 3.8 to 8.1, with a resolution of 0.2-0.3, in accordance with the manufacturer's instructions (Macherey-Nagel, Germany). For buffer capacity, the final pH was measured after 10 minutes mixing 1 ml saliva with 3 ml of 0.005 M HCl.

Bacterial counts: Caries associated bacteria, *Streptococcus mutans and Lactobacillus* spp. were determined using the spatula method (Kohler and Bratthall 1979). A sterile wooden spatula was pressed against a disposable contact petri dish (Nunc, Denmark) with Mitis-Salivarius-Bacitracin (MSB) agar for *S.mutans* enumeration and Rogosa SL agar for *Lactobacillus* spp. The agar plate was placed in a candle jar and transported to the laboratory within 3 hours after the specimen had been collected. It was then incubated at 37^{o}C in the candle jar for 48 hours. The number of colonies was microscopically counted for mutans streptococci, and visually counted for *Lactobacillus* spp.

Fluoride: Fluoride is an important protective factor for dental caries. Besides fluoride toothpaste or fluoride supplement, fluoride levels in drinking water from various primary sources in the community was collected and assessed using a fluoride electrode (Orion 710 Aplus, Sciencetech Co., Ltd.).

Statistical analysis: Double data entry was performed into EpiData 2.0 ("The EpiData Association", Odense, Denmark) to validate the accuracy of data entry. Afterwards the data were transferred by

Stat Transfer 6 for analysis in the Stata statistical program version 7.0. The mutans streptococci quantity was finally grouped as <100 and >100 CFU. These levels are equal to $<10^6$ and $>10^6$ CFU/ml of saliva (Westergren and Krasse 1978). *Lactobacillus* spp. counts were categorized as <10, >10 corresponding to $<10^4$ and $>10^4$ CFU/ml in saliva. (Pongpaisan et al. 2000). A distribution of blood lead levels was rather highly right-skewed, it was therefore normalized by making a log arithmetic transformation. DMFS and dfs for each child were computed from the examination results. The Spearman rank correlation coefficient (Rs) was computed between PbB and dmfs, and PbB and DMFS, using original values of PbB. However the association may be plausibly confounded by other factors. A multiple logistic regression model was used to examine the association. The very high prevalence of dental caries in deciduous teeth (95%) implies that almost all children had at least one surface of deciduous caries. Therefore the prediction of dfs>5 surfaces was considered to compare the different levels of caries experience. For covariates, bivariate analysis was performed to explore the associations with high and low caries. Variables at the level of $p \leq 0.2$, based on chi-square (for categorical data) and Kruskal Wallis test /Mann-Whitney U-test (for non-normally distributed continuous data) were added to the model. With PbB kept for all models, likelihood-ratio tests were performed on each of other variables. Variables the likelihood-ratio tests were significant at the level of $p \leq 0.05$ or known to be important risk factors for caries were retained.

Results

A total of 292 children completed clinical oral examinations and blood lead determination. Twenty children had no deciduous teeth and three children had no permanent teeth. This left 272 children for analysis of deciduous teeth and 289 children for analysis of permanent teeth. The geometric mean for PbB and SD were 7.2 and 1.5 μg/dl, respectively. Sixty children (21%) had a lead exposure level of more than 10 μg/dl. The children in high and low PbB groups revealed similar socio-economic status. None of the variables in Table 1 had significant association with the PbB level. Most of the children (82%) were Muslims. The majority of the children's parents finished primary school and 37% of families had an income of more than 100,000 baht/year. Overall most children had high consumption of sugar-containing sweets. Thirty-three percent of children brushed their teeth less than once a day and most guardians had never helped their children brush their teeth. Almost all (88%) used fluoride toothpaste.

Most residents in the community primarily consumed both artesian well water and the municipal water supply. The fluoride levels in those sources ranged from 0.04 to 0.15 ppm. This reflects low fluoride exposure in the drinking water compared with the CDC recommended concentration of 0.7-1.2 ppm for supplementation (CDC 2001).

Table 2 shows very high prevalence and severity of caries. The mean for DMFS and dfs were 1.3 and 13.2 surfaces/child respectively. The dfs of children with a PbB level equal to or over 10 μg/dl was significantly higher than the low blood lead group. The oral hygiene was generally poor as demonstrated by high levels of plaque and calculus. Saliva flow rate was generally normal, but there was significantly high saliva acidity among the high blood lead group. Overall, children who had either high or low PbB had a high colony count of *S.mutans*. Sixty-one percent of all children had a *S.mutans* count of more than 50 CFU/1.5 cm^2. In Figure1, the gross relationships between PbB and caries indices are displayed using a multiple box plot, cutting PbB into four groups at 2, 4, 8 and 16 μg/dl. When blood lead level increases, dfs tends to increase (Rs=0.25, p=0.00). However it should be noted that the increasing trend stops after PbB is above 16μg/dl, although the number of subjects in this group was small. The dose response relationship with DMFS was not significant (Rs=0.09, p=0.14).

From logistic regression results in Table 3, blood lead level was not an important predictor for DMFS≥1 (OR=1.28, p=0.35), whereas age (p<0.01), religion (OR=0.38, p<0.01), and high plaque levels (p<0.01) were significant factors. In Table 4, blood lead level was confirmed to be a caries associating factor for deciduous teeth (OR=2.39, p<0.01) after adjusting for other factors. When PbB is doubled, the odds for having dfs>5 increases by 2.39 times. Also age, high *lactobacillus* spp. counts (>10^4 CFU/ml) and low education of father were risk factors for dfs.

Discussion

Our studied population has a relatively low level of socio-economic development and poor oral behaviour in addition to a prevalence (21%) of PbB ≥10 μg/dl. There is no evidence of association between the socio-economic variables and PbB. The level of dfs, but not DMFS was associated with the blood lead level. The relationships are confirmed with multivariate analysis with adjustment for age, gender, socio-economic status, oral health behaviours, oral hygiene, the number of teeth present, salivary parameters, and cariogenic bacteria.

In the study population of this age group, deciduous teeth had long been in the oral cavity but the permanent teeth had just erupted. The number of tooth surfaces at risk and subsequently the prevalence of dental caries in deciduous teeth were higher than those of permanent teeth. The power of our study is enough to detect the cariogenic effect of lead in deciduous teeth (74%), but not in permanent teeth (14%).

The dose-response relationship between PbB and deciduous caries risk defined as >5 surfaces in our study is strong (doubling PbB increases the odds by 2.39 times). This is in contrast to the finding of low and weak association by Campbell et al. (2000) and inconsistent associations (only in urban but not in rural areas) by Gemmel et al. (2002). The age groups in all these studies are comparable (6-10/6-12 years old). However, the mean PbB were the highest in our study. The degree of caries in our study was also higher (dfs=13.2, SD=9.6) than in the others (Gemmel's study: 8.0±

6.5; Campbell's study: 3.4). Being at higher risk may allow higher power of the study to test the hypothesis in our study.

Our finding of no significant association between PbB and DMFS is consistent with the above two previous studies (Campbell et al. 2000; Gemmel et al. 2002). All three studies were carried out on schoolchildren whose number and duration of permanent teeth at risk were low. The association in permanent teeth may be found when the children get older, as reported in the study by Moss et al. (1999). Among 21,354 studied subjects of 5-17 years of age, where there were comparisons of caries-free children vs. those with some caries (DMFS≥1), the OR for each 5 μg/dl change in blood lead level was 1.8 (95%CI=1.3-2.5).

Fluoridation of water supply has been proved to prevent dental caries (Levy 2003; Petersen and Lennon 2004). The fluoride level in drinking water in our studied population was low. Area coverage of fluoride supplementation was poor but the use of fluoride toothpaste was common. The fluoride program in this area was not effective enough, as the prevalence of caries was still high and there was no association found between exposure to the fluoride program and a reduced risk of caries. We did not find significant interaction between PbB and the use of fluoride toothpaste. Neither did the previous desalivated rat study by Tabchoury et al. (1999) find that the protective effect of fluoride in drinking water was reduced by the presence of Pb. With our limited knowledge of the human system, there is a need for a controlled experiment in high PbB populations of young children to refine the interaction effect with fluoride with respect to its various routes of administration.

Our data do not conclusively support the hypothetical mechanism of lead cariogenicity through a decreased saliva flow rate. In contrast, Watson et al. (1997) reported that offspring rats with exposure to Pb in utero had a salivary flow rate 30% less than the control group. Later on, the same group of investigators (Watson et al. 1999) contrastly demonstrated that pre- and perinatal exposure to lead in rats had no effect on salivary gland weight and the Ca^{2+}–activated Cl^{-} channel. We found no difference in secretion rates between high lead-exposed and low lead-exposed children. Perhaps the dose in animal experiment by Watson et al. was much higher (48 μg/dl in pregnancy) than the natural exposure levels in most human populations. Species difference could also, in part, account for the discrepancy.

The effect of Pb at the pre-eruptive vs. post-eruptive stage on subsequent caries is not clear. Grobler et al. (2000) and Purchase and Fergusson (1986) reported higher lead concentration in dentine than in enamel and the highest lead concentration was in the area of circumpulpal dentine. A decline in lead away from the pulp cavity (Purchase and Fergusson 1986) and the equivalent levels of lead content in individual match-paired impacted and erupted third molars as researched by Bercovitz and Laufer (1992), suggest that Pb enters the tooth from the blood stream. However, higher lead content was found in surface enamel than in the enamel-dentine junction, implying that part of the lead accumulation in enamel may be acquired at the post-eruptive period from the oral environment (Brudevold et al. 1977; Purchase and Fergusson 1986). Therefore, the incorporation of lead into the tooth structure may be implicated in a reduced resistance of enamel to dental caries. Gerlach et al. (2002) showed that regions of maturing enamel were not as hard in mice exposed to lead as in control animals. The results of deciduous caries in the present finding is more likely to be the combination of the effect of pre- and post-eruptive lead exposure, due to long duration of exposure to the oral environment. In contrast, pre-eruptive lead exposure is assumed to mainly account for permanent caries of this age group.

The effect of Pb on caries in the human population is not as strong and consistent as that in experimental animals. Apart from species differences, this observation may be explained by fluctuation of PbB in most human subjects. PbB reflects recent exposure that may vary over time. Using a single point measurement of PbB to relate to caries risk probably underestimates the cariogenic effect of average long-term PbB because of the regression dilution effect (Knuiman 1998).

In summary, in this shipyard area, lead exposure is a potential factor in the development for deciduous caries after adjusting for various other factors, but the evidence for its relation with permanent caries is still unconfirmed due to the short duration and small number of permanent teeth. Children living in high risk areas with lead exposure should therefore be given priority to access intensive caries control programs given that lead exposure cannot be controlled easily. An extended study to precisely verify the long-term cariogenic effect of lead exposure on permanent teeth is still needed.

Acknowlegements and Grant information: This work was supported by the Thailand Research Fund (TRF, PHD/0175/2542, BGJ4580015) and the Commission on Higher Education, the Swedish foundation for International Cooperation in Research and Higher Education (STINT), and the Swedish International Development Cooperation Agency (SIDA). The Epidemiology Unit is partially supported by the TRF and Thai Health Promotion Fund through a Senior Research Scholar Grant for Professor Virasakdi Chongsuvivatwong. I wish to thank subjects and their parents, schoolteachers and health officers in the study area for their kindly cooperation, and Nipa Maharachpong for helps in introducing to the local community surrounding the shipyards.

References

Anderson RJ, Davies BE, James PM. Dental caries prevalence in a heavy metal contaminated area of the West of England. Br.Dent.J 1976 ; 141: 311-314.

Anderson RJ, Davies BE, Nunn JH, James PM. The dental health of children from five villages in North Somerset with reference to environmental cadmium and lead. Br.Dent.J 1979; 147: 159-161.

Bercovitz K, Laufer D. Systemic lead absorption in human tooth roots. Arch.Oral Biol 1992; 37: 385-387.

Bloch P, Shapiro IM, Soule L, Close A, Revich B. Assessment of lead exposure of children from K-XRF measurements of shed teeth. Appl.Radiat.Isot 1998; 49: 703-705.

Brudevold F, Aasenden R, Srinivasian BN, Bakhos Y. Lead in enamel and saliva, dental caries and the use of enamel biopsies for measuring past exposure to lead. J.Dent.Res 1977; 56: 1165-1171.

Campbell JR, Moss ME, Raubertas RF. The association between caries and childhood lead exposure. Environ.Health Perspect 2000; 108: 1099-1102.

Centers for Disease Control and Prevention (CDC). Preventing lead poisoning in young children. Public health statement by the Centers for Disease Control. US. Department of Health and Human Services. 1991.

Centers for Disease Control and Prevention (CDC). Fluoride recommendations for government to protect human health. Public health statement by the Centers for Disease Control. US. Department of Health and Human Services. 2001.

Davies BE, Anderson RJ. The epidemiology of dental caries in relation to environmental trace elements. Experientia 1987; 43: 87-92.

Department of Health. The fifth national oral health survey. Ministry of Public Health. Bangkok. 2000-2001.

Geater A, Duerawee M, Chompikul J, Chairatanamanokorn S, Pongsuwan N, Chongsuvivatwong V, et al. Blood lead levels among schoolchildren living in the Pattani River Basin: two contamination scenarios? J. Environ. Med 2000; 2: 11-16.

Gemmel A, Tavares M, Alperin S, Soncini J, Daniel D, Dunn J, et al. Blood lead level and dental caries in school-age children. Environ.Health Perspect 2002; 110: A625-A630.

Gerlach RF, Cury JA, Krug FJ, Line SR. Effect of lead on dental enamel formation. Toxicology 2002; 175: 27-34.

Gil F, Facio A, Villanueva E, Perez ML, Tojo R, Gil A. The association of tooth lead content with dental health factors. Sci.Total.Environ 1996; 192: 183-191.

Goldman LR. Children--unique and vulnerable. Environmental risks facing children and recommendations for response. Environ.Health Perspect 1995; 103(suppl-6):13-18.

Greene JC, Vermillion JR. The simplified oral hygiene index. J Am Dent Assoc 1964; 68: 7-13.

Grobler SR, Rossouw RJ, Kotze TJ, Stander IA. The effect of airborne lead on lead levels of blood, incisors and alveolar bone of rats. Arch Oral Biol 1991; 36: 357-360.

Grobler SR, Theunissen FS, Kotze TJ. The relation between lead concentrations in human dental tissues and in blood. Arch Oral Biol 2000; 45: 607-9.

Karakaya A, Ilko M, Ulusu T, Akal N, Isimer A, Karakaya AE. Lead levels in deciduous teeth of children from urban and suburban regions in Ankara (Turkey). Bull.Environ.Contam.Toxicol 1996; 56: 16-20.

Knuiman MW, Divitini ML, Buzas JS, Fitzgerald PE. Adjustment for regression dilution in epidemiological regression analyses. Ann.Epidemiol 1998; 8: 56-63.

Kohler B, Bratthall D. Practical method to facilitate estimation of Streptococcus mutans levels in saliva. J.Clin.Microbiol 1979; 9: 584-588.

Levy SM. An update on fluorides and fluorosis. J Can Dent Assoc 2003; 69: 286-91.

Moss ME, Lanphear BP, Auinger P. Association of dental caries and blood lead levels. JAMA 1999; 281: 2294-2298.

Petersen PE, Lennon MA. Effective use of fluorides for the prevention of dental caries in the 21st century: the WHO approach. Community Dent Oral Epidemiol 2004; 32: 319-21.

Pongpaisan S, Teanpaisan R, Ngoenwiwatkul Y, Narksawat K. Modified spatula method to estimate lactobacilli levels in saliva: application for community dentistry. J. Dent. Assoc. Thai. 2000; 50: 468-471.

Provincial Health Authority of Pattani and Faculty of Medicine, Prince of Songkla Univeristy. Lead poisoning of shipyard workers in Pattani Province, Thailand. Internal report 2000.

Purchase NG, Fergusson JE. Lead in teeth: the influence of the tooth type and the sample within a tooth on lead levels. Sci.Total.Environ 1986; 52: 239-250.

Schwartz J. Low-level lead exposure and children's IQ: a meta-analysis and search for a threshold. Environ.Res 1994; 65: 42-55.

Selypes A, Banfalvi S, Bokros F, Gyory E, Takacs S. Chronic lead exposure in children living in Miskolc, Hungary, on the basis of teeth lead levels. Bull.Environ.Contam.Toxicol 1997; 58: 408-414.

Steenhout A. Kinetics of lead storage in teeth and bones: an epidemiologic approach. Arch.Environ.Health 1982; 37: 224-231.

Tabchoury CM, Pearson SK, Bowen WH. Influence of lead on the cariostatic effect of fluoride co-crystallized with sucrose in desalivated rats. Oral Dis 1999; 5:100-103.

Tvinnereim HM, Eide R, Riise T, Wesenberg GR, Fosse G, Steinnes E. Lead in primary teeth from Norway: changes in lead levels from the 1970s to the 1990s. Sci.Total.Environ 1997; 207: 165-177.

Watson GE, Davis BA, Raubertas RF, Pearson SK, Bowen WH. Influence of maternal lead ingestion on caries in rat pups. Nat.Med 1997; 3: 1024-1025.

Watson GE, Evans RL, Melvin JE, Pearson SK, Bowen WH. Influence of lead exposure on salivary gland function in rats. J Dent Res 1999; 78: 340.

Westergren G, Krasse B. Evaluation of a micromethod for determination of Streptococcus mutans and Lactobacillus infection. J.Clin.Microbiol 1978; 7: 82-83.

World Health Organization. Oral Health Surveys, basic methods. 4th ed. The Oral Health Programme. Switzerland. 1997.

Table1: Distribution of demographic characteristics ,socio-economic status, oral health behaviour, and dietary habit of children

Variable	All children n (%)	Children with blood lead level<10μg/dl	Children with blood lead level ≥10μg/dl
Age (years, mean±SD,)	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)
Religion			
Buddhist	53 (18)	38 (16)	15 (25)
Muslim	239 (82)	194 (84)	45 (75)
Socio-economic status			
Level of father's education			
No education	7 (2)	6 (3)	1 (2)
Primary school	202 (73)	161 (73)	41 (73)
Secondary school	35 (13)	27 (12)	8 (14)
Occupational college/vocational	7 (2)	6 (3)	1 (2)
Level of mother's education			
No education	10 (3)	10 (4)	0 (0)
Primary school	233 (82)	181 (80)	52 (91)
Secondary school	28 (10)	26 (11)	2 (3)
Occupational college/vocational	2 (1)	2 (1)	0 (0)

Table 1 (continue)

Variable	Total N (%)	Children with blood lead level <10μg/dl	Children with blood lead level ≥10μg/dl
Housing status			
Concrete/concrete-half	207 (71)	163 (70)	44 (73)
Wooden/corrugated iron	83 (28)	67 (29)	16 (27)
Poor/ temporary house	2 (1)	2 (1)	0 (0)
Family income (baht/year)			
< 50,000	52 (18)	37 (16)	15 (25)
50,001-80,000	92 (32)	75 (33)	17 (28)
80,001-100,000	39 (13)	35 (15)	4 (7)
> 100,000	107 (37)	83 (36)	24 (40)
Child's Oral health behaviour			
Frequency of tooth-brushing			
< once a day	96 (33)	76 (33)	20 (33)
once a day	71 (24)	57 (25)	14 (23)
≥ twice a day	125 (43)	99 (43)	26 (43)
Frequency of dental visit			
Never/<once a year	101 (35)	78 (34)	23 (38)
≥ once a year	191 (65)	154 (66)	37 (62)
Tooth cleaning by guardian			
Never	209 (72)	166 (71)	43 (72)
Rare/ sometimes	83 (28)	66 (28)	17 (28)

Table 1 (continue)

Variable	Total N (%)	Children with blood level level <10μg/dl	Children with blood lead level ≥10μg/dl
Fluoride toothpaste			
Non-fluoride	35 (12)	26 (11)	9 (15)
Fluoride	255 (88)	206 (89)	49 (84)
Fluoride supplement			
Never	166 (57)	128 (55)	38 (64)
Irregular	112 (38)	95 (41)	17 (29)
Regular	13 (4)	9 (4)	4 (7)
Use of additional tooth-cleaning equipment			
No	243 (83)	193 (83)	50 (83)
Yes	49 (17)	39 (17)	10 (17)
Frequency of sugar-containing sweets			
< twice a day	56 (19)	46 (20)	10 (17)
twice a day	136 (47)	110 (47)	26 (43)
3-4+ times a day	100 (34)	76 (33)	24 (40)

Table2: Distribution of caries and caries associated factors

Variable	All children (n=292) Mean ±SD(range)	Children with blood lead level <10μg/dl (n=232)	Children with blood leadlevel ≥10μg/dl (n=60)
Number of caries free children (n=292)	9/292 (3.1%)	9/232 (3.9%)	0/60 (0%)
Caries prevalence in permanent teeth	126/ 289 (43.6%)	96/229 (41.9%)	30/60 (50%)
Caries prevalence in deciduous teeth*	257/272 (94%)	200/215 (93%)	57/57 (100%)
Tooth surface present (n=292)	100.7±8.0 (66-128)	101.3±7.6 (76-128)	98.7±9.0 (66-108)
Permanent surface present (n=289)	58.2 ±22.2 (0-128)	58.9±22.8 (0-128)	55.7±19.3 (20-108)
Deciduous surface present (n=272)	46.3±20.3 (0-88)	46.6±20.7 (0-88)	45.3±19.2 (0-84)
DMFS (n=289)	1.3±2.3 (0-17)	1.2±2.3 (0-17)	1.5±2.4 (0-14)
dfs** (n=272)	13.2 ±9.6 (0-45)	12.3±8.9 (0-36)	16.5±11.3 (1-45)
Plaque n (%)			
Mild	55 (19)	47 (20)	8 (13)
Moderate	139 (48)	110 (47)	29 (48)
Heavy	98 (34)	75 (32)	23 (38)
Calculus n (%)			
No	179 (61)	144 (62)	35 (58)
Mild	83 (28)	63 (27)	20 (33)
Moderate & heavy	30 (10)	25 (11)	5 (8)
Salivary parameters			
Flow rate (ml/min)	0.8 ±0.4 (0.2-2.8)	0.8±0.4 (0.2-2.8)	0.7±0.5 (0.2-2.1)
pH**	7.3 ±0.3 (6.3-8.1)	7.3±0.3 (6.3-8.1)	7.1±0.3 (6.5-7.5)
Buffer capacity	6.5 ±0.2 (5.5-7.2)	6.5±0.2 (5.5-7.2)	6.5±0.3 (5.7-6.9)
Cariogenic bacteria			
Mutans streptococci (CFU) n (%)			
0	2 (1)	1 (0)	1 (2)
1-10	23 (8)	15 (6)	8 (13)
11-50	90 (31)	74 (32)	16 (27)
51-99	70 (24)	58 (25)	12 (20)
100+	107 (37)	84 (36)	23 (38)
Lactobacillus spp.(CFU) n (%)			
0	96 (33)	78 (34)	18 (30)
1-10	98 (34)	74 (32)	24 (40)
11-50	75 (26)	62 (27)	13 (22)
51-99	11 (4)	9 (4)	2 (3)
100+	12 (4)	9 (4)	3 (5)

* Statistically significant difference: p-value<0.05

** Statistically significant difference: p-value<0.01

Table 3: Multiple logistic regression model predicting DMFS≥1 (n=289)

Variable	Category	OR	95%CI	P-value*
Doubling of blood lead level $Log_2(PbB)$		1.28	0.81-2.04	0.35
Age group (years)	6-7	1		0.00
	8	0.92	0.36-2.36	
	9	2.32	0.90-5.98	
	10-11	3.60	1.24-10.44	
Gender	boy	1		0.62
	girl	1.06	0.62-1.81	
Religion	Buddhist	1		0.00
	Muslim	0.38	0.19-0.77	
Plaque	mild	1		0.00
	moderate	3.12	1.44-6.78	
	heavy	5.34	2.31-12.30	
Number of permanent tooth surfaces present		1.02	1.00-1.04	0.02

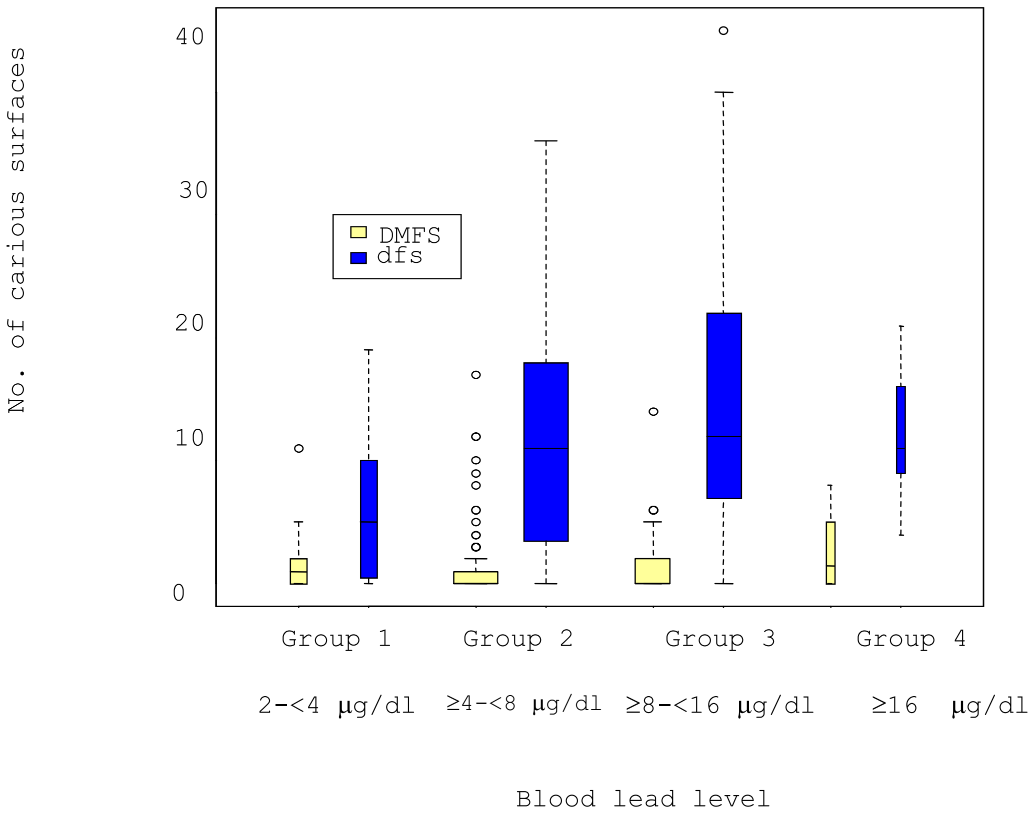
* P-value from likelihood-ratio test for difference among all groups

Table 4: Multiple logistic regression model predicting dfs>5 (n=272)

Variable	Category	OR	95%CI	P-value*
Doubling of blood lead level $\log_2$(PbB)		2.39	1.36-4.20	0.004
Age group (years)	6-7	1		0.02
	8	0.79	0.28-2.26	
	9	2.36	0.74-7.53	
	10-11	1.58	0.46-5.43	
Gender	boy	1		0.79
	girl	1.14	0.61-2.15	
Father's education	primary school	1		0.00
	secondary school-or higher	0.87	0.75-0.99	
Number of deciduous tooth surfaces present		1.05	1.03-1.07	0.00
Lactobacilli spp. count (CFU/ml in saliva)	$<10^4$	1		0.00
	$>10^4$	2.69	1.30-5.56	

* P-value from likelihood-ratio test for difference among all groups

Figure 1: DMFS and dfs in relation to blood lead level



Note : Box width reflects relative sample size in the particular group.

2. MANUSCRIPT 2

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

Nattaporn Youravong[1], Rawee Teanpaisan[3], Virasakdi Chongsuvivatwong[1], Alan F. Geater[1], Gunnar Dahlén[2]

[1] Epidemiology Unit, Faculty of Medicine

Prince of Songkla University, Hatyai, Songkhla, Thailand 90112

[2]Department of Oral Microbiology

Sahlgrenska Academy at Göteborg University, Göteborg, Sweden box 450 SE 405 30

[3]Department of Stomatology

Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand 90112

Abstract

The purpose of this study was to evaluate the prevalence and the level of selected oral bacterial species in association with dental caries in low lead-exposed versus high lead-exposed children. With an observational cross-sectional design, a sample of 292 children aged 6-11 years from two primary schools around a shipyard area, 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 method. Enumeration of 17 bacterial species was carried out using the checkerboard DNA-DNA hybridization technique. *Lactobacillus acidophilus* counts were significantly lower in high lead-exposed children compared to those of the low lead-exposed group ($p<0.01$). The level of *Veillonella parvula* was significantly higher ($p<0.01$) in children with a high blood lead (PbB) level. *V. parvula* was also associated with high dfs in children with low PbB. Other associations between the levels of bacterial species and caries experience (DMFS and dfs) in children with high blood lead levels in comparison with those with normal blood lead levels were of no or little significance. Conclusively, a lower level of *Lactobacillus* spp. in the dental plaque of children with high blood lead levels might be explained by either direct or indirect effect of lead on bacteria, however the relation to caries development remains unclear.

Introduction

Lead (Pb) is a major environmental pollutant and a hazard to health. Adverse health effects of lead exposure in children are well-documented (Goldman, 1995; Wolfe, 2000). One of its main targets is the nervous system (Wolfe, 2000). Meta-analysis revealed a highly significant association between blood lead levels and IQ in school-aged children (Schwartz, 1994). An association between lead exposure and higher caries prevalence has been also suggested, even those few published studies so far have shown controversial results (Brudevold *et al.*, 1977; Gil *et al.*, 1996; Moss *et al.*, 1999; Campbell *et al.*, 2000; Bowen, 2001; Gemmel *et al.*, 2002). Our previous report has demonstrated a significantly higher number of decayed and filled deciduous surfaces in high lead-exposed compared to low lead-exposed children (Youravong *et al.*, 2005a). However, the mechanisms behind lead influence on caries development remain unclear. Mineralization of the enamel could be altered due to a delay in enamel maturation (Gerlach *et al.*, 2002), which may increase the susceptibility of the tooth to dental caries process. However, no morphological changes connected to lead in blood could be found in decidious teeth of these children (Youravong *et al.*, 2005b) Secondly, administration of lead significantly (30-40 %) can diminish stimulated salivary flow rates (Craan *et al.*, 1984; Watson *et al.*, 1997) which may have an indirect influence on the bacterial activity and plaque formation rate. Thirdly, lead may also have a direct effect on bacteria that may result in alterations in the activity of some bacterial species and subsequently the composition of the oral microflora. Schamschula *et al.* (1978) found that elevated level of lead in plaque was associated with increased prevalence of caries however the microflora was not studied. Gil *et al.* (1996) documented high numbers of salivary lactobacilli and mutans streptococci in a group of individuals with high lead level in teeth. It has also been suggested that the assessment of lactobacilli, mutans streptococci and other bacterial species should be determined in the dental plaque for a better relevance to caries than saliva (Sanchez-Perez & Acosta-Gio, 2001; Seki *et al.*, 2003).

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

Materials and methods

Study setting

The study area was a shipyard area which was 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 (Watbosub School and Banhuakhao School) located in the area, were enlisted and asked for parent's informed consent before oral examination of all children. Only children having informed consent, no major health problem, and continuous residence in the area were invited to participate in this study. Four milliliters of venous blood was drawn from the cubital vein of the subject and sent for analysis of blood lead level (PbB) using Atomic Absorption Spectrophotometer (GFAAS, HITACHI Model Z-8200). The children with a blood lead level above 10 μg/dl were considered as high lead-exposed according to Centers for Disease Control (CDC 1991). Clinical oral examination was conducted to define carious lesions and oral status according to the criteria of World Health Organization (1997) using the simplified oral health record form. 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.

Bacterial plaque sample

Plaque samples were collected by toothbrush method (Okada *et al.*, 2000). A new toothbrush (6-9-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 then 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 then transferred into an Eppendorf tube and stored at -20°C until futher processing.

Checkerboard DNA-DNA hybridization

The analysis of bacterial species was performed using the checkerboard DNA-DNA hybridization method at the laboratory of Oral Microbiology, Göteborg University, Sweden. Digoxigenin-labeled, whole genomic DNA probes were prepared using the High-Prime labelling kit (Boehringer-Mannheim, Germany). Genomic DNA of bacterial species were *Streptococcus mutans* (ATCC 25175), *Streptococcus sanguis* (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, and immobilized by UV light and incubated at 120ºC for 20 minutes. After 2 hours of prehybridization, the standard DNA probes were allowed to hybridize overnight with the sample DNA using a second blotting device at 42ºC. After low and high stringency washes at 65ºC, 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 of species. The signals were coded on a scale from 0-5, where 0 indicates no signal; 1, a signal density weaker than that of the low standard (i.e, < 10^5 bacteria); 2, signal density equal to that of the low standard (=10^5 bacteria); 3, signal density higher than that of the low standard but lower than that of the high standard (>10^5 but <10^6 bacteria); 4, signal density equal to that of the high standard (=10^6 bacteria); 5, signal density higher that 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 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 recategorized as 3 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 lead-exposed and low lead-exposed children, the data were dichotomized ($<10^6$ and $\geq 10^6$ cells). Breakdown of means of dfs and DMFS were carried out by level of each bacterial count ($<10^6$ and $\geq 10^6$ cells) and stratified by level of lead exposure (PbB<10 μg/dl vs. PbB ≥10 μg/dl). In each stratum, Wilcoxon rank-sum test was used to examine the effect of bacterial count on dfs and DMFS.

Results

A number of 292 children aged 6-11 years were completed clinical oral examination, blood lead level and bacterial determination. Twenty children had only permanent teeth and three children 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 1. The two groups of lead-exposed children had similar oral hygiene and total number of present tooth surfaces. The mean number of decayed and filled deciduous surfaces (dfs) was significantly higher in high lead-exposed children than low lead-exposed children ($p<0.01$), whereas the mean of decayed, missing, and filled permanent surfaces (DMFS) was not significantly different between the two groups.

The distribution of bacteria in different levels in the two groups of children is demonstrated in Table 2. High prevalent and predominating species (over 10^6 cells) included *A. naeslundii* (100%), *S. sanguis* (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 2, the level of *L. acidophilus* was lower in high lead-exposed children than in low lead-exposed group. *V. parvula* on the other hand showed a higher level in the low lead-exposed children ($p<0.01$). In Table 3, in an unstratified analysis, high counts of *V. parvula* and *F. nucleatum* were significantly associated with increased mean dfs number ($p<0.01$ and 0.05 respectively). After stratification by PbB level, significant association was found in only low PbB group of *V. parvula*. No bacterial species was significantly associated with mean dfs number in high lead-exposed group.

For permanent teeth (Table 4), *A. actinomycetemcomitans* was negatively associated with the mean DMFS number, and this association was confined to the group of low lead-exposed children. No bacterial species was significantly associated with a mean DMFS number in high lead-exposed group.

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, two showed an association with PbB level. The level of *L. acidophilus* was lower whereas the number of *V. parvula* was higher in the group of lead-exposed children. With regard to dental caries in the deciduous dentition, high levels of *V. parvula* and *S. noxia* were associated with high dfs, especially in low lead-exposed children. For dental caries in the permanent dentition, only *A. actinomycetemcomitans* was negatively associated with the caries and the relation was confined to the low lead-exposed group.

High bacterial levels for some of streptococcal species and *Actinomyces naeslundii* in this study is not surprising in view of the collected plaque was supragingival (Marsh, 1994). The high levels may also have the other reason such as the utilization of toothbrush sample for collecting pooled supragingival plaque instead of a saliva sample. Saliva was not used due to the observation (unpublished) that saliva from certain individuals does not stick to the nylon membranes in the checkerboard method. However, the use of toothbrush samples instead of sampling plaque with a curette may probably overload the sample with bacteria and the upper detection limit is exceeded in the checkerboard DNA-DNA assay. The checkerboard assay also contributes as most molecular based methods to an increase in prevalence for the majority of species, as compared to conventional culture technique, particularly fastidious periodontal pathogens (Papapanou *et al.*, 1997). Both *P. gingivalis* and *T. forsythia* were detected less frequently by culture than either with immunofluorescence or with checkerboard technique (Tanner *et al.*, 1998). The quality of the template DNA may also affect the sensitivity and specificity of the findings (Wall-Manning *et al.*, 2002). A lower specificity of the checkerboard technique can occur in heavy plaque samples (van Steenbergen *et al.*, 1996; Papapanou *et al.*, 1997) and in this respect the toothbrush samples should be avoided in patients with poor oral hygiene and high plaque scores. Thus, statistical calculations was not performed on *A. naeslundii, S. mutans, S. sanguis,* 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. sanguis, S. oralis*, and *Actinomyces* spp. which could be found in oral cavity of children as early as infancy or predentate period (Nyvad & Kilian, 1990; Pearce *et al.*, 1995; Caufield *et al.*, 2000; Kamma *et al.*, 2000b; Könönen, 2000). They play an important role in supragingival plaque formation on both decidious and permanent teeth (Marsh and Martin, 1999). Other organisms generally regarded as late colonizers (Marsh and Martin, 1999) such as *Fusobacterium* spp., *Prevotella* spp., *E. corrodens*, and *C. ochracea*, are reported to be common in children (Könönen *et al.*, 1994; Frisken *et al.*, 1990; Kamma *et al.*, 2000a; Goldstein *et al.*, 1983), and the high prevalence of these 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. An increased prevalence than previously thought have been recorded also for these bacteria using molecular biology techniques (for review see Darby and Curtis, 2001). Conclusively, no apparent difference was found in the dental plaque flora in these children from southern Thailand from what could be expected from other populations.

A number of bacteria such as mutans streptococci and lactobacilli are known to be associated with caries and suggested to indicate active caries or a high caries risk (Nyvad and Kilian, 1990; Hardie, 1992; Ansai *et al.*, 1994; Marsh, 1994; Marsh and Martin, 1999; Botha *et al.*, 2001). More caries was recorded for the deciduous teeth in the high PbB group but not for their permanent teeth (Table 1). 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 caries (DMFS) can not be expected in this age group (Youravong *et al.*, 2005a). On the other hand the children had significantly developed more caries in the deciduous teeth (dfs) and therefore higher levels of both mutans streptococci and lactobacilli in the high lead level group were expected. This association has also been documented for children with high lead levels in their teeth (Gil *et al.*, 1996). However, no association between the dfs values in these children versus mutans streptococci and/or lactobacilli in saliva was found in the previous study (Youravong *et al.*, 2005a). 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 lead level group (Table 3), suggesting a limited relation to caries. This perhaps unexpected finding, could be explained by different methods of specimen collection (e.g. saliva vs. plaque) and the specific detection of *L. acidophilus* in this study, instead of the total lactobacillus counts as in the previous (Youravong *et al.*, 2005a).

We also found *V. parvula* associated with caries in deciduous teeth. This species, however, was reported by Russell *et al.* (1990) to have no association with caries in low-risk individuals. The association found in our study may be explained by the ability of *V. parvula* to enhance acid-producing activity of *S. mutans* (Mikx & Van der Hoeven, 1975; Hardie, 1992), which was found abundant among our subjects. This enhancement activity may be less active in the high blood lead group.

Of several bacteria examined in our sample, the associations with caries are less common in high PbB than in low PbB children. A lower level of lactobacilli in the dental plaque of children with high blood lead levels could be explained by a direct or indirect effect on the bacterial activity. However, the higher caries development of children with a high blood lead level remains unsolved. It is worthwhile to confirm this effect modification of lead with further in vitro studies.

Acknowledgements and Grant information: This work was supported by the Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. program (grant No. PHD/0175/2542, BGJ 4580015), the Commission on Higher Education of Thailand, the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), the Swedish Research Council (Vetenskapsrådet), and the Swedish International Development Cooperation Agency (SIDA). We would like to thank Susanne Blomqvist, laboratory staff at Department of Oral Microbiology, Faculty of Odontology, Göteborg University, Sweden, for her kind technical assistance.

References

1. **Ansai, T., Yamashita, Y., Shibata, Y., Katoh, Y., Sakao, S., Takamatsu, N., Miyazaki, H. & Takehara, T. (1994).** Relationship between dental caries experience of a group of Japanese kindergarten children and the results of two caries activity tests conducted on their saliva and dental plaque. *Int J Paediatr Dent* **4**, 13-17.

2. **Botha, F.S., Botha, S.J. , Kroon, J. & Steyn, P.L. (2001).** Caries prediction factors in children with primary dentition. *SADJ* **56**, 348-352.

3. **Bowen, W.H. (2001).** Exposure to metal ions and susceptibility to dental caries. *J Dent Educ* **65**,1046-1053.

4. **Brudevold, F., Aasenden, R., Srinivasian, B.N. & Bakhos, V. (1977).** Lead in enamel and saliva, dental caries and the use of enamel biopsies for measuring past exposure to lead. *J Dent Res* **56**, 1165-1171.

5. **Campbell, J.R., Moss, M.E. & Raubertas, R.F. (2000).** The association between caries and childhood lead exposure. *Environ Health Perspect* **108**, 1099-1102.

6. **Caufield, P.W., Dasanayake, A.P. Li, Y., Pan, Y., Hsu, J. & Hardin, J.M. (2000).** Natural history of Streptococcus sanguinis in the oral cavity of infants: evidence for a discrete window of infectivity. *Infect Immun* **68**, 4018-4023.

7. Centers for Disease Control (CDC). (1991). Preventing lead poisoning in young children. A statement by the Centers for Disease Control. US Department of Health and Human Services.

8. Craan, A.G., Nadon, G.& P'an, A.Y. (1984). Lead flux through the kidney and salivary glands of rats. *Am J Physiol* **247,** F773-F783

9. Darby, I. & Curtis, M. (2001). Microbiology of periodontal disease in children and young adults. *Periodontology 2000* **26,** 33-53

10. Frisken, K.W., Higgins,T. & Palmer, J.M. (1990). The incidence of periodontopathic microorganisms in young children. *Oral Microbiol Immunol* **5,** 43-45.

11. Gemmel, A., Tavares, M., Alperin, S., Soncini, J., Daniel, D., Dunn, J., Crawford, S., Braveman, N., Clarkson, T.W., McKinlay, S. & Bellinger, D.C. (2002). Blood lead level and dental caries in school-age children. *Environ Health Perspect* **110,** A625-A630

12. Gerlach, R.F., Cury, J.A., Krug, F.J. & Line, S.R. (2002). Effect of lead on dental enamel formation. *Toxicology* **175,** 27-34.

13. Gil, F., Facio, A., Villanueva, E., Perez, M.L., Tojo, R. & Gil, A. (1996). The association of tooth lead content with dental health factors. *Sci Total Environ* **192,** 183-191.

14. Goldman, L.R. (1995). Children--unique and vulnerable. Environmental risks facing children and recommendations for response. *Environ Health Perspect* **103 Suppl 6,** 13-18.

15. Goldstein, E.J.C., Tarenzi, L.A., Agyare, E.O. & Berger, J.R. (1983). Prevalence of *Eikenella corrodens* in dental plaque. *J Clin Microbiol* **17**, 636-9.

16. Hardie, J.M. (1992). Oral microbiology: current concepts in the microbiology of dental caries and periodontal disease. *Br Dent J* **172**, 271-278.

17. Kamma, J.J., Diamanti-Kipioti, A., Nakou, M. & Mitsis, F.J. (2000a). Profile of subgingival microbiota in children with mixed dentition. *Oral Microbiol Immunol* **15**, 103-111.

18. Kamma, J.J., Diamanti-Kipioti, A., Nakou, M. & Mitsis, F.J. (2000b). Profile of subgingival microbiota in children with primary dentition. *J Periodontal Res* **35**, 33-41.

19. Könönen, E., Asikaianen, S., Saarela, M., Karjalainen, J. & Jousimies-Somer, H. (1994). The oral gramnegative anaerobic microflora in young children: longitudinal changes from edentolous to dentate mouth. *Oral Microbiol Immunol* **9**, 136-141.

20. Könönen, E. (2000). Development of oral bacterial flora in young children. *Ann Med* **32**, 107-112.

21. Marsh, P.D. (1994). Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res* **8**, 263-271.

22. Marsh, P.D. & Martin, M. (1999). Oral Microbiology. 4th edn. Wright. Oxford UK

23. Mikx, F.H.M. & Van der Hoeven, J.S. (1975). Symbiosis of Streptococcus mutans and Veillonella alcalescens in mixed continuous cultures. *Arch Oral Biol* **20,** 407-410.

24. Moss, M.E., Lanphear, B.P. & Auinger, P. (1999). Association of dental caries and blood lead levels. *JAMA* **281,** 2294-2298.

25. Nyvad, B. & Kilian, M. (1990). Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. *Caries Res* **24,** 267-272.

26. Okada, M., Hayashi, F. & Nagasaka, N. (2000). Detection of Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in dental plaque samples from children 2 to 12 years of age. *J Clin Periodontol* **27,** 763-768.

27. Papapanou, P.N., Madianos, P.N., Dahlen, G. & Sandros, J. (1997). "Checkerboard" versus culture: a comparison between two methods for identification of subgingival microbiota. *Eur J Oral Sci* **105,** 389-396.

28. Pearce, C., Bowden, G.H., Evans, M., Fitzsimmons, S.P., Johnson, J., Sheridan, M.J., Wientzen, R. & Cole, M.F. (1995). Identification of pioneer viridans streptococci in the oral cavity of human neonates. *J Med Microbiol* **42,** 67-72.

29. Russell, J.I., MacFarlane, T.W., Aitchison,T.C., Stephen, K.W. & Burchell, C.K. (1990). Caries prevalence and microbiological and salivary caries activity tests in Scottish adolescents. *Community Dent Oral Epidemiol* **18,** 120-125.

30. Sanchez-Perez, L. & Acosta-Gio, A.E. (2001). Caries risk assessment from dental plaque and salivary Streptococcus mutans counts on two culture media. *Arch Oral Biol* **46**, 49-55.

31. Schamschula, R.G., Bunzel, M., Agus, H.M., Adkins, B.L., Barmes, D.E. & Charlton, G. (1978). Plaque minerals and caries experience: associations and interrelationships. *J Dent Res* **57**, 427-432.

32. Schwartz, J. (1994). Low-level lead exposure and children's IQ: a meta-analysis and search for a threshold. *Environ Res* **65**, 42-55.

33. Seki, M., Karakama, F., Terajima, T., Ichikawa, Y., Ozaki, T., Yoshida, S. & Yamashita, Y. (2003). Evaluation of mutans streptococci in plaque and saliva: correlation with caries development in preschool children. *J Dent* **31**, 283-290.

34. Tanner, A.C.R., Maiden, M.F.J., Zambon, J.J., Thoren, G.S. & Kent, R.L.Jr (1998). Rapid chair-side DNA probe assay of Bacteroides forsythus and Porphyromonas gingivalis. *J Periodontal Res* **33**,105-117.

35. van Steenbergen, T.J. M., Timmerman, M.F., Mikx, F.H.M., de Quincey, G., van der Weijden, G.A., van der Velden, U. & de Graaff, J. (1996). Discrepancy between culture and DNA probe analysis for the detection of periodontal bacteria. *J Clin Periodontol* **23**, 955-959.

36. Wall-Manning, G.M.; Sissons, C.H.; Anderson, S.A. & Lee, M. (2002). Checkerboard DNA-DNA hybridisation technology focused on the analysis of Gram-positive cariogenic bacteria. *J Microbiol Methods* **51**, 301-311.

37. Watson, G.E., Davis, B.A., Raubertas, R.F., Pearson, S.K. & Bowen, W.H. (1997). Influence of maternal lead ingestion on caries in rat pups. *Nat Med* **3**, 1024-1025.

38. Wolfe, C.A. (2000). Pediatric alert: assess for lead. *RN* **63**, 26-30.

39. World Health Organization. (1997). Oral Health Surveys, basic methods. 4th edn. The Oral Health Programme. Switzerland.

40. Youravong, N., Chongsuvivatwong, V., Geater, A.F., Dahlén, G. & Teanpaisan, R. (2005a). Lead associated caries development in children living in a lead-contaminated area, Thailand. *Sci Total Environ,* in press.

41. Youravong, N., Chongsuvivatwong, V., Teanpaisan, R., Geater, A.F., Dietz, W., Dahlén, G. & Norén J. (2005b). Morphology of enamel in primary teeth from children in Thailand exposed to environmental lead. *Sci Total Environ* **348**, 73-81.

Table 1: Characteristics and clinical parameters of children

Variable	All children (n=292) n (%) or mean(SD)	Children with blood lead level <10μg/dl n=232	Children with blood lead level ≥10μg/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 & heavy	30 (10)	25 (11)	5 (9)

* Statistically significant difference: p-value<0.01, Wilcoxon rank-sum test

Table 2 Distribution of bacterial count from toothbrush sample by blood lead level of the study subjects.

Bacterial species	Children with blood lead<10μg/dl(n (%))			Children with blood lead ≥10μg/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. viscosus	- (0)	- (0)	232 (100)	- (0)	- (0)	60 (100)	NA
S. sanguis	- (0)	- (0)	232 (100)	- (0)	- (0)	60 (100)	NA
S. mutans	- (0)	1 (1)	231 (99)	- (0)	- (0)	60 (100)	0.61
S. intermedius	- (0)	2 (1)	230 (99)	- (0)	- (0)	60 (100)	0.47
S. oralis	- (0)	6 (3)	226 (97)	- (0)	1 (2)	59 (98)	0.68
E. corrodens	- (0)	10 (4)	222 (96)	- (0)	1 (2)	59 (98)	0.34
C. ochracea	14 (6)	15 (6)	203 (88)	- (0)	4 (7)	56 (93)	0.15
P. nigrescens	22 (9)	55 (24)	155 (67)	5 (8)	11 (18)	44 (74)	0.61
P. intermedia	20 (9)	89 (38)	123 (53)	4 (7)	26 (43)	30 (50)	0.74
V. parvula	4 (2)	181 (78)	47 (20)	1 (2)	36 (60)	23 (38)	0.01*
A. actinomycetemcomitans	3 (1)	195 (84)	34 (15)	1 (2)	48 (80)	11 (18)	0.75
F. nucleatum	2 (1)	21 (91)	18 (8)	0 (0)	50 (83)	10 (17)	0.09
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)	0.44

Table 2 (continue)

Bacterial species	Children with Blood lead<10μg/dl(n (%))			Children with Blood lead ≥10μg/dl (n(%))			P-value
	≤10^5	10^5 -10^6	≥10^6	≤10^5	10^5 -10^6	≥10^6	
S. noxia	56 (24)	175 (75)	1 (1)	12 (20)	48 (80)	- (0)	0.69
T. forsythensis	85 (37)	146 (63)	1 (0)	19 (32)	41 (68)	- (0)	0.67
C. rectus	186 (80)	46 (19)	- (0)	53 (88)	7 (12)	- (0)	0.14

Statistically significant difference: p-value<0.05*, <0.01**, Chi square test

NA denotes not available due to the fact that all sample had ≥ 10^6 cells

Table 3: Mean (SD) of number of decayed and filled deciduous surfaces (dfs) in relation to different bacterial levels in high lead-exposed children and low lead-exposed children (n=272).

Bacteria	dfs					
	All children		Low lead-exposed children		High lead-exposed children	
	$<10^6$	$\geq 10^6$	$<10^6$	$\geq 10^6$	$<10^6$	$\geq 10^6$
A. viscosus	NA	NA	NA	NA	NA	NA
S. sanguis	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]	12(5.43)	13.21 (9.66)	10.25 (4.35)	12.35 (8.99)	19 (-)	16.46 (11.36)
E. corrodens[b]	18.18 (11.85)	12.98 (9.46)	17.20 (12.02)	12.07 (8.72)	28 (-)	16.30 (11.26)
C. ochracea	13.38 (8.89)	13.16 (9.69)	12.00 (7.39)	12.35 (9.12)	22.00 (13.64)	16.09 (11.11)
P. nigrescens	12.21 (10.05)	13.62 (9.38)	11.29 (9.59)	12.79 (8.58)	16.47 (11.30)	16.52 (11.39)
P. intermedia	12.02 (8.66)	14.21 (10.26)	11.50 (8.61)	13.01 (9.17)	13.96 (8.74)	18.8 (12.85)
V. parvula	12.12** (9.28)	16.66** (9.86)	11.35** (8.54)	16.02** (9.52)	15.67 (11.62)	18.05 (10.69)
A.actinomycetemcomitans	13.21 (9.62)	13.07 (9.56)	12.37 (8.91)	11.93 (9.11)	16.37 (11.49)	17.22 (10.59)
F. nucleatum	12.69* (9.30)	17.70* (11.13)	11.97 (8.87)	15.94 (8.99)	15.62 (10.51)	21.22 (14.49)
L. acidophilus[a]	11.96 (7.96)	13.31 (9.75)	10.61 (8.24)	12.41 (8.97)	13.42 (7.74)	17.33 (11.97)
P. gingivalis[a]	12.74 (9.76)	13.69 (9.42)	11.29 (8.62)	13.52 (9.17)	19.00 (11.91)	14.27 (10.35)

Table 3 (continue)

Bacteria	dfs					
	All children		Low lead-exposed children		High lead-exposed children	
	<10^6	≥10^6	<10^6	≥10^6	<10^6	≥10^6
S. noxia[a]	11.48 (9.50)	13.69 (9.59)	10.22* (8.71)	12.96* (8.92)	17.36 (11.18)	16.30 (11.40)
T. forsythensis[a]	12.14 (9.50)	13.74 (9.63)	11.05 (8.59)	12.99 (9.06)	16.72 (11.84)	16.41 (11.15)
C. rectus[a]	13.17 (9.86)	13.27 (8.45)	12.25 (9.29)	12.53 (7.46)	16.30 (11.14)	18.00 (13.00)

* Statistically significant difference at p-value<0.05, Wilcoxson rank-sum test

** Statistically significant difference at p-value<0.01, Wilcoxson rank-sum test

[a] Strains were categorized into ≤10^5 and >10^5 cells.

[b] Strains were not tested for difference in high lead-exposed group due to too high percentage of ≥10^6 cells.

NA denotes mean and sd. not available due to the fact that all sample had ≥ 10^6 cells

Table 4: Mean (SD) of number of decayed and filled permanent surfaces (DMFS) in relation to different bacterial levels in high lead-exposed children and low lead-exposed children (n=289).

	dfs					
Bacteria	All children		Low lead-exposed children		High lead-exposed children	
	$<10^6$	$\geq10^6$	$<10^6$	$\geq10^6$	$<10^6$	$\geq10^6$
A. viscosus	NA	NA	NA	NA	NA	NA
S. sanguis	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]	2.14 (3.58)	1.24 (2.30)	2.17 (3.92)	1.17 (2.26)	2.00 (-)	1.54 (2.46)
E. corrodens[b]	1.09 (1.45)	1.27 (2.37)	0.80 (1.13)	1.21 (2.35)	4.00 (-)	1.51 (2.44)
C. ochracea	1.30 (3.04)	1.26 (2.24)	1.48 (3.20)	1.15 (2.16)	0 (0)	1.66 (2.49)
P. nigrescens	1.16 (2.02)	1.32 (2.48)	1.12 (2.05)	1.23 (2.44)	1.37 (1.89)	1.61 (2.62)
P. intermedia	1.48 (2.53)	1.07 (2.13)	1.38 (2.41)	1.02 (2.21)	1.87 (2.93)	1.23 (1.81)
V. parvula	1.21 (2.28)	1.45 (2.52)	1.20 (2.40)	1.14 (1.90)	1.24 (1.57)	2.04 (3.39)
A.actinomycetemcomitans	1.34 (2.34)	0.83 (2.30)	1.32** (2.44)	0.35** (0.88)	1.41 (1.92)	2.18 (4.09)
F. nucleatum	1.20 (2.17)	1.89 (3.54)	1.20 (2.29)	1.11 (2.65)	1.20 (1.60)	3.30 (4.57)
L. acidophilus[a]	2.10 (3.08)	1.17 (2.22)	2.71 (3.77)	1.07 (2.12)	1.31 (1.65)	1.62 (2.62)
P. gingivalis[a]	1.34 (2.33)	1.17 (2.35)	1.18 (2.10)	1.21 (2.57)	2.07 (3.07)	1.03 (1.45)

Table 4 (continue)

Bacteria	dfs					
	All children		Low lead-exposed children		High lead-exposed children	
	$<10^6$	$\geq10^6$	$<10^6$	$\geq10^6$	$<10^6$	$\geq10^6$
S. noxia[a]	1.33 (2.38)	1.25 (2.33)	1.29 (2.49)	1.16 (2.26)	1.50 (1.88)	1.56 (2.58)
T. forsythensis[a]	1.27 (2.14)	1.26 (2.45)	1.14 (2.16)	1.22 (2.40)	1.84 (1.98)	1.41 (2.64)
C. rectus[a]	1.36 (2.45)	0.83 (1.68)	1.32 (2.44)	0.67 (1.61)	1.51 (2.52)	1.86 (1.86)

* Statistically significant difference at p-value<0.05, Wilcoxson rank-sum test

[a] Strains were categorized into $\leq10^5$ and $>10^5$ cells.

[b] Strains were not tested for difference in high lead-exposed group due to too high percentage of $\geq10^6$ cells.

NA denotes mean and sd. not available due to the fact that all sample had $\geq 10^6$ cells

3. MANUSCRIPT 3

Morphology of enamel in primary teeth from children in Thailand exposed to environmental lead

Nattaporn Youravong[a], Virasakdi Chongsuvivatwong[a], Rawee Teanpaisan[b], Alan F. Geater[a], Wolfram Dietz[c], Gunnar Dahlén[d], Jörgen G. Norén[e]

[a]Epidemiology unit, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand.

[b]Department of Stomatology, Faculty of Dentistry, Prince of Songkla University, Songkhla, Thailand.

[c]Centre of Electron Microscopy, Friedrich-Schiller-University Jena, Germany

[d]Department of Oral Microbiology, Faculty of Odontology, Göteborg University, Sweden.

[e]Department of Pedodontics, Faculty of Odontology, Göteborg University, Sweden.

This manuscript was published in *the Science of the Total Environment* 2005; 348: 73-81.

Abstract

Lead is one of the major environmental pollutants and a health risk. Dental hard tissues have a capacity to accumulate lead from the environment. Eighty exfoliated primary teeth were collected from children residing around a shipyard area in southern Thailand, known for its lead contamination. The morphology of the enamel was examined by polarized light microscopy, microradiography (MRG), and scanning electron microscopy. The specimens derived from two groups of children, one group with high blood levels of lead (57 teeth) and one group having low blood levels of lead (23 teeth). The enamel irrespective of group appeared normal. However, in a majority of the specimens the enamel surface appeared hypomineralized, which was confirmed in SEM. No morphological changes connected to lead in blood could be found. The hypomineralized surface zone could possibly be attributed to an acid oral environment.

Keywords: Lead; primary teeth; enamel; hypomineralization, enamel hypoplasia

Introduction

Lead is a potentially hazardous health risk in industrial areas and large cities. In a number of studies it has been demonstrated that dental hard tissues have the capacity to accumulate lead and other heavy metals from the environment (Fosse and Justesen 1978; van Wyk and Grobler 1983; Haavikko et al. 1984; Anttila and Anttila 1987; Anttila 1986; Anttila 1987a; Anttila 1987b; Frank et al. 1988; Heilmann et al. 1990; Frank et al. 1990; Cleymaet et al. 1991a; Cleymaet et al. 1991b; Cleymaet et al. 1991c; Cleymaet et al. 1991d; Cleymaet et al. 1991e). In a Belgian study, a positive correlation between exposure to lead and an enamel lead concentration in the surface enamel was found (Cleymaet et al. 1991b).

Heavy metals are known to incorporate into dental hard tissues both during their mineralization and also posteruptively. Enzymes involved in the extracellular matrix metabolism are inhibited by excess heavy-metal ions. Lead, as well as cadmium and zinc, may inhibit the activity of enamel matrix proteinases, even in low concentrations, thereby interfering with the amelogenesis (Gerlach et al. 2000). It appears that lead affects the development of teeth and other organs, not least the neurological ones. In teeth, lead may increase susceptibility to caries. One explanation could be that enamel hardness decreases due to a delay in enamel maturation (Gerlach et al. 2002). Lead has been found in both enamel and dentine, though the levels were higher in enamel (Knychalska-Karwan et al. 1985). In a Finnish study, it was shown that the concentration of lead was higher on the buccal side as compared with the lingual (Anttila and Anttila 1987). In previous studies, an association between exposure to lead and higher caries prevalence has been shown (Derise et al. 1974; Bowen 2001).

In the Singhanakhon District of southern Thailand, a higher prevalence of caries has been noted among children living close to small shipyards where environmental contamination of lead is known (Youravong et al. 2004). A relationship between living near shipyards and increased lead levels in blood serum indicates a possible uptake of lead in dental hard tissues and also an increased risk for caries.

The aim of this study was, by means of polarization microscopy (PLM) and scanning electron microscopy (SEM), to examine the morphology of enamel of primary teeth in children with high levels of lead in serum and compare with a group of children with low levels of lead in serum.

Material and methods

2.1 Subjects

The patients live in the Singhanakhon District, Songkhla Province of southern Thailand. The Huakhao subdistrict consists of 8 villages, where income sources are fishing and boat repair in small shipyards. This area is known to be endemic for lead exposure. Lead has contaminated both soil and dust in this area. The shipyard industry uses Pb_3O_4 in repairs. Three primary schools are located in the subdistrict; Watbosub School, Banhuakhao School, and Bankhaodang School. Two of the schools are located near the shipyards. Teeth were collected from children attending these two schools, after they and their parents consented to the study. The project and the consent form were approved by the ethic committee, Faculty of Medicine, Prince of Songkla University.

A detailed background to caries and blood lead-levels is presented in a clinical paper. Therefore, only a brief description of the children (age range 6-10 years) represented by their primary teeth will be given here (Youravong et al., 2005). The number of children with blood lead-levels <10 μg/dl is 57 and for levels ≥10 μg/dl the number is 23 (Table 1). For the original study population (292 children) the **df** (**d**=decayed; **f**=filled; primary teeth) values were significantly higher ($p<0.05$) in the high blood lead-level group compared with the low level group (Youravong et al. 2005). However no statistically significant difference was found among subjects submitting their teeth for this study (Table 1).

2.2 Tooth material

All 292 children attending the Watbosub and Banhuakhao Schools were asked to hand in one exfoliated tooth each. Eighty exfoliated primary teeth thus, bacame eligible-the teeth were stored dry until they were prepared for the morphological examinations. Twenty-three of these teeth originated from children who had been exposed to lead and were known to have high levels of lead in their serum. The remaining 57 teeth originated from children who had low blood lead levels (Table 1). Prior to the histological and chemical investigations, all teeth were macroscopically examined for caries, enamel aberrations and other findings.

The teeth were oriented and embedded in an epoxy resin (Epofix®, Electron Microscopy Sciences, Fort Washington, USA) and serially sectioned in bucco-lingual direction to a thickness of 100-120 μm with an Exakt diamond band saw (Exakt®, Exakt Vertriebs GMBH, Norderstedt, Germany). Central sections were used for the microscopic investigations.

2.3 Polarized light microscopy

All sections were examined in polarized light, both dry-in-air as well as after water imbibition for 24 hours, in an Olympus polarizing microscope employing strainfree objectives. The PLM examination was carried out without knowledge of which group the specimen belonged to.

2.4 Scanning electron microscopy

Seven sections from the Pb-group and five from the control group were selected for the SEM analysis. The selection was based on the PLM examination and the sections represented the various morphological findings. The sections were etched with 30 % phosphoric acid, carefully rinsed with distilled water, mounted on sample holders for the microscope, sputtered with gold, and investigated in a Philips SEM 515 at 15kV. Two intact non-etched teeth from each group were also coated with gold, by vapour deposition, and the enamel surface was examined in SEM.

2.5 Statistical analysis

Morphological differences of the pre- and post-natally formed enamel in hypomineralization and enamel hypoplasia as seen in PLM between high and low blood lead groups were compared using Pearson’s chi-square or Fisher’s exact test.

3. Results

3.1 Macroscopical examination

The macroscopical examination of the teeth revealed that in the control group (no. 57), 22 teeth exhibited caries, 23 showed yellow staining of the enamel and 9 teeth had enamel hypoplasias with a diameter of approximately 1-1.5 mm. In the Pb group (no. 23), 10 teeth had caries, 12 yellow staining. In two teeth, enamel hypoplasias were found. No statistical significant differences of these distributions between the groups were found.

3.2 Polarized light microscopy

All specimens were first examined dry-in-air. In 50/57 tooth sections from the control group, and in 17/23 teeth from the Pb-group, the neonatal line could readily be discerned. The neonatal line appeared as a distinct, positively birefringent band extending from the enamel-dentine junction in the most cervical part, towards the enamel surface at the incisal part of the tooth (Fig. 1A). The enamel along the line exhibited a pore volume distribution of 5% or more as the line remained positively birefringent after water imbibition. In 6 and 5 teeth in the two groups, respectively, no neonatal line could be seen. In the control group, three teeth were so abraded that only the cervical enamel was left and in the Pb-group, one severely abraded tooth was found. In the cases where the neonatal line could be found, it became possible to distinguish between prenatally and postnatally formed enamel.

The prenatal primary enamel appeared positively birefringent in all sections, indicating a higher degree of porosity in the tissue (Fig. 1A). After water imbibition, this micro porous zone changed to negatively birefringence in 28/57 of the control teeth. In 26 control specimens, however, this zone remained positively birefringent, indicating a pore volume distribution of more than 5%. In two of the specimens, the enamel was regarded to have extremely low mineralization. The corresponding figures for the Pb-group were 11/23 with a normal degree of mineralization of the prenatal enamel, while 11 had a lower degree than normal and one specimen exhibited a very low degree of mineralization (Fig. 1B).

The postnatal enamel appeared negatively birefringent except for those cases where the neonatal line was positioned more toward the thin incisal/cuspal part of the enamel. The inner microporous zone was observed extending over the neonatal line into the postnatally formed enamel (Fig. 1B). The postnatal enamel appeared normal in 45/57 control specimens and 17/23 Pb-group specimens. A lower degree of mineralization than normal was found in 11 control specimens and in five from the Pb-group. In each group, one specimen was regarded as having a very low degree of mineralization. Specimens with both pre- and postnatally low degrees of mineralization were found in each group, 8 and 4 respectively.

In a majority of the specimens, 48/57 and 16/23, a distinct hypomineralization of the enamel surface was seen (Fig. 1C). The surface zone appeared as a positively birefringent zone with a width of approximately 10 μm. In these teeth no normal highly mineralized surface zone could be found. The zone appeared as a homogenous band along the surface from the incisal part of the tooth to the cervical area.

In the postnatal enamel a hypomineralized zone, deep to the enamel surface, could be seen in 2/57 of the control sections and in 2/23 of the sections from the Pb-teeth (Fig. 1D). In 15/57 of the control teeth initial caries was also found, mainly in the cervical area. The corresponding figure for the Pb-group was 6/23 sections with caries (Fig. 2A). However, in some cases, caries could be seen directly in relation to the hypomineralized zone.

In three of the control teeth and three of the Pb-group teeth, enamel hypoplasias were found. The enamel hypoplasias were located along the neonatal line. The cervical border of the defect was smooth and rounded and the prisms bent perpendicular to the rounded surface. No other aberrations of the enamel were found in connection with the enamel hypoplasias. In one case, three consecutive hypoplasias were seen (Fig. 2B).

Other more pronounced incremental lines than the neonatal line were found prenatally in two control teeth and five Pb-group teeth. In the postnatal enamel of 12 teeth in the control group and three in the Pb-group, more pronounced incremental lines were found.

3.3 Microradiography

The micrographs confirmed the findings in PLM with the hypomineralized character of the neonatal line and the pre- and postnatally formed enamel that appeared hypomineralized. In normal enamel, the surface zone appears in MRG as a more radioopaque band. In the specimens with a demineralized surface zone the outer surface appeared less distinct.

3.4 SEM

All specimens, seven in the Pb-group and five in the control group, were thoroughly examined in SEM. As the surrounding epoxy resin had loosened from the surface of six of the specimen, it thus became possible to examine these enamel surfaces as well. The general morphology did not differ between the controls and the Pb-specimens.

Irrespective of group, the enamel surface of the teeth which in PLM appeared to have an etched-like hypomineralized zone, no aprismatic outer surface could be discerned (Fig. 3A). This was more evident in the cervical half of the crown. Compared to normal enamel, the ends of the prisms were seen directly perpendicular to the enamel surface. In the more incisal parts of the crowns, the prisms had bent perpendicularly to their original direction and were seen lying flat on the surface. In all sections exhibiting a porous surface, prisms could easily be detected. This is, to some extent, an effect of etching, however, in normal enamel, a more porous surface can be seen but with a still mainly intact surface. In low magnification, the surface near the hypomineralized zone appeared dark in SEM with a depth between 10-20 μm. In higher magnification, a distinct difference between the dark zone and underlying enamel was found (Figs. 3B and C). In the normal underlying enamel, prisms were arranged in a structured way and the crystallites were even and regular in shape, size and arrangement. In the dark area, the structure appeared with less contrast and the crystallites were less well arranged, with differences in size and shape. Pores in the enamel were also found. Additionally, the boundary between the dark zone and the rather normal structured enamel was characterized by a step at the section surface (Figs. 3A and D). This step is a result of a preparation causing shrinkage of less mineralized material within the dark zone.

In connection with the hypomineralized band, a more pronounced hypomineralized zone was observed in PLM, was seen in the postnatal enamel and sometimes also in the more cervical parts with initial caries lesions (Fig. 12). The hypomineralized area in SEM showed a less pronounced prism structure and an irregular arrangement of prisms and crystallites. The difference in morphology between the hypomineralized area and the initial caries was mainly an increase of pores in the enamel of the caries lesion, and the prisms and crystallites were still fairly even and regularly arranged (Figs. 4A and B).

In the sections where no dark band could be seen in SEM, the surface layer still had a lack of aprismatic surface layer. The SEM examination revealed that all teeth had an enamel surface which evidently had been exposed to an acid environment in the oral cavity. Furthermore, no distinct differences could be discerned between teeth from children exposed to Pb and those that were not.

The enamel hypoplasias as seen in SEM showed that no normal surface layer existed in the bottom of the defect. At the surface, prisms were readily seen exhibiting an irregular pattern. As seen in the PLM, the cervical border of the defect was rounded, however, with less regular prism structure. The prisms were regularly arranged prenatally to the neonatal line, but the postnatally located enamel prisms, deep to the cervical border, appeared more irregular (Figs. 4C, D, and E).

4. Discussion

This study has shown that there were no morphological differences seen in PLM, MRG or SEM in the enamel between teeth from a group of children with high blood lead-levels and a group with low blood lead-levels. The main finding, however, was a porous surface enamel layer, most noticeable on the buccal side of the teeth.

The rate of participation among children with high blood lead levels was only marginally higher than that of those having low blood lead levels. However, although it was not possible to analyze the reason for not handing in exfoliated teeth, there is no reason to believe that there would be any significant differences between those participating and those who are not.

The findings in this tooth material did not differ from what has previously been found in morphological studies of the enamel of primary teeth. The presence of a neonatal line in the enamel is in agreement with findings in Swedish studies (Norén 1983; Ranggård et al. 1994). The microporous inner zone of the prenatal enamel is a normal finding in primary teeth and appears to have a certain extension. In some cases, where the neonatal line is positioned more incisively (shorter gestational age), it extends into the postnatal enamel (Norén 1983). Only few specimens had a very low degree of mineralization in this zone, possibly indicating a minor disturbance in the maturation phase of the enamel. Nor did the postnatally formed enamel appear to have structural or other changes in either of the groups.

A well mineralized surface was only found in a few cases; instead a large part of the enamel surface appeared hypomineralized. There are good reasons to believe that this is a result of an acid oral environment. The caries data for the individuals in this study confirm that caries prevalence is high. A limited exposure to fluorides, a diet with cariogenic factors, and an inadequate oral hygiene may contribute to the occurrence of this hypomineralized zone. This is strengthened by the macroscopical findings of orange staining which is a sign of proper oral hygiene (Shafer 1983). The SEM analyses confirmed the hypomineralized character of the surface enamel, showing no aprismatic outer enamel layer.

In previous studies of primary teeth, a postnatally located subsurface hypomineralized zone has been demonstrated to occur frequently (Norén 1983). In this study this zone was found only in a few specimens, which can be explained by the hypomineralized surface disguising it. However, in SEM, a hypomineralized zone was found with less pronounced enamel structure. It differed from caries lesions since they showed an increase in pores, especially at the surface.

The numbers of enamel hypoplasias are somewhat high; however, as the number of patients is limited compared with the original population, no conclusions can be drawn. Their appearance was in agreement with other studies (Norén 1983). An increase in enamel hypoplasias in children exposed to high blood lead-levels has been shown (Lawson et al. 1971), but as all but one enamel hypoplasia had a chronology connected to birth, there is no reason to assume that there is a direct connection to lead exposure in these cases.

It can be concluded that no differences in enamel morphology could be seen between those specimens coming from children with low blood lead-levels and those having high levels. In a rat study, where the effect of lead on enamel formation was investigated, no macroscopical changes could be seen other than indications of altered mineralization (Gerlach et al. 2002). A relative increase in the amount of protein was detected, possibly resulting in a decrease in the microhardness of the rat enamel (Gerlach et al. 2002). The exposure to lead of the investigated population evidently has not been on such levels that morphological changes of the primary enamel have occurred.

Acknowledgements

This study was supported by Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. Grant (grant No. PHD/0175/2542, BGJ4580015) and the Commission on Higher Education, Ministry of Education, and the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), The Swedish Research Council (Vetenskapsrådet).

References

Anttila A. Proton-induced X-ray emission analysis of Zn, Sr and Pb in human deciduous tooth enamel and its relationship to dental caries scores. Arch Oral Biol 1986; 31: 723-6.

Anttila A, Anttila A. Trace-element content in the enamel surface and in whole enamel of deciduous incisors by proton-induced X-ray emission of children from rural and urban Finnish areas. Arch Oral Biol 1987; 32: 713-7.

Anttila A. Concentrations of lead and some other trace elements in deciduous teeth measured by proton-induced X-ray emission. Proc Finn Dent Soc 1987a; 83: 1-64.

Anttila A. Lead content of deciduous tooth enamel from a high-radon area. Acta Odontol Scand 1987b; 45: 283-8.

Bowen WH. Exposure to metal ions and susceptibility to dental caries. J Dent Educ 2001; 65: 1046-53.

Cleymaet R, Bottenberg P, Retief DH, Slop D, Michotte Y, Coomans D. In vivo use of a dual acid etch biopsy for the evaluation of lead profiles in human surface enamel. Caries Res 1991a; 25: 256-63.

Cleymaet R, Bottenberg P, Slop D, Clara R, Coomans D. Study of lead and cadmium content of surface enamel of schoolchildren from an industrial area in Belgium. Community Dent Oral Epidemiol 1991b; 19: 107-11.

Cleymaet R, Collys K, Retief DH, Michotte Y, Slop D, Taghon E, Maex W, Coomans D. Relation between lead in surface tooth enamel, blood, and saliva from children residing in the vicinity of a non-ferrous metal plant in Belgium. Br J Ind Med 1991c; 48: 702-9.

Cleymaet R, Quartier E, Slop D, Retief DH, Smeyers-Verbeke J, Coomans D. Model for assessment of lead content in human surface enamel. J Toxicol Environ Health 1991d; 32: 111-27.

Cleymaet R, Retief DH, Quartier E, Slop D, Coomans D, Michotte Y. A comparative study of the lead and cadmium content of surface enamel of Belgian and Kenyan children. Sci Total Environ 1991e; 104: 175-89.

Derise NL, Ritchey SJ, Furr AK. Mineral composition of normal human enamel and dentin and the relation of composition to dental caries. I. Macrominerals and comparison of methods of analyses. J Dent Res 1974; 53: 847-52.

Fosse G, Justesen NP. Lead in deciduous teeth of Norwegian children. Arch Environ Health. 1978; 33: 166-75.

Frank RM, Sargentini-Maier ML, Leroy MJ, Turlot JC. Age-related lead increase in human permanent teeth demonstrated by energy dispersive X-ray fluorescence. J Trace Elem Electrolytes Health Dis 1988; 2: 175-9.

Frank RM, Sargentini-Maier ML, Turlot JC, Leroy MJ. Comparison of lead levels in human permanent teeth from Strasbourg, Mexico City, and rural zones of Alsace. J Dent Res 1990; 69: 90-3.

Gerlach RF, Cury JA, Krug FJ, Line SR. Effect of lead on dental enamel formation. Toxicology 2002; 175: 27-34.

Gerlach RF, de Souza AP, Cury JA, Line SR. Effect of lead, cadmium and zinc on the activity of enamel matrix proteinases in vitro. Eur J Oral Sci 2000; 108: 327-34.

Haavikko K, Anttila A, Helle A, Vuori E. Lead concentrations of enamel and dentine of deciduous teeth of children from two Finnish towns. Arch Environ Health 1984; 39: 78-84.

Heilmann HH, Kuhl K, Steckhan F, Steckhan F. Gradienten anorganischer Bestandteile im Schmelz und Dentin von Milchzahnen. II. Vergleich der Konzentrationen von Zink, Magnesium, Kalzium und Blei. Zahn Mund Kieferheilkd Zentralbl 1990; 78: 587-92.

Knychalska-Karwan Z, Pawlicki R, Karwan T. Quantitative, qualitative and topographic distribution of lead, zinc, iron and copper in hard teeth tissues. Folia Histochem Cytobiol 1985; 23: 21-5.

Lawson BF, Stout FW, Ahern DE, Sneed WD. The incidence of enamel hypoplasia associated with chronic pediatric lead poisoning. South Carolina Dent J 1971; 29: 5-10.

Norén JG. Enamel structure in deciduous teeth from low-birth-weight infants. Acta Odontol Scand 1983; 41: 355-62.

Ranggård L, Norén JG, Nelson N. Clinical and histologic appearance in enamel of primary teeth in relation to neonatal blood ionized calcium values. Scand J Dent Res 1994; 102: 254-9.

Shafer WG. A textbook of oral pathology. Philadelphia: Saunders 1983. p. 710.

van Wyk CW, Grobler SR. Lead levels in deciduous teeth of children from selected urban areas in the Cape Peninsula. S Afr Med J 1983; 63: 559-62.

Youravong N, Chongsuvivatwong V, Geater AF, Dahlén G, Teanpaisan, R. Lead associated caries development in children living in a lead contaminated area, Thailand. *Sci Total Environ* 2005, in press.

Table 1. The mean number of decayed and filled deciduous teeth (dft) and surfaces (dfs) by the blood lead-level group (μg/dl).

Blood lead-level (μg/dl)	dfs Mean± SD (Range)	dft Mean± SD (Range)	Frequency
<10	9.7±7.5 (0-31)	3.7±2.5 (0-11)	56[a]
≥10	12.8±9.3 (2-36)	4.5±2.7 (1-11)	23
Total	10.6±8.1(0-36)	3.9±2.6 (0-11)	79

[a] One child in high blood lead group (1/57) without deciduous teeth was excluded for calculation of dfs and dft, but included for tooth analysis.

Table 2. Distribution of examined teeth from the low blood lead level group and high level, respectively.

	Maxilla			**Mandible**	
	Incisors	Canines	Molars	Incisors	Canines
<10 μg/dl	17	9	4	10	17
≥10 μg/dl	6	9	1	3	4

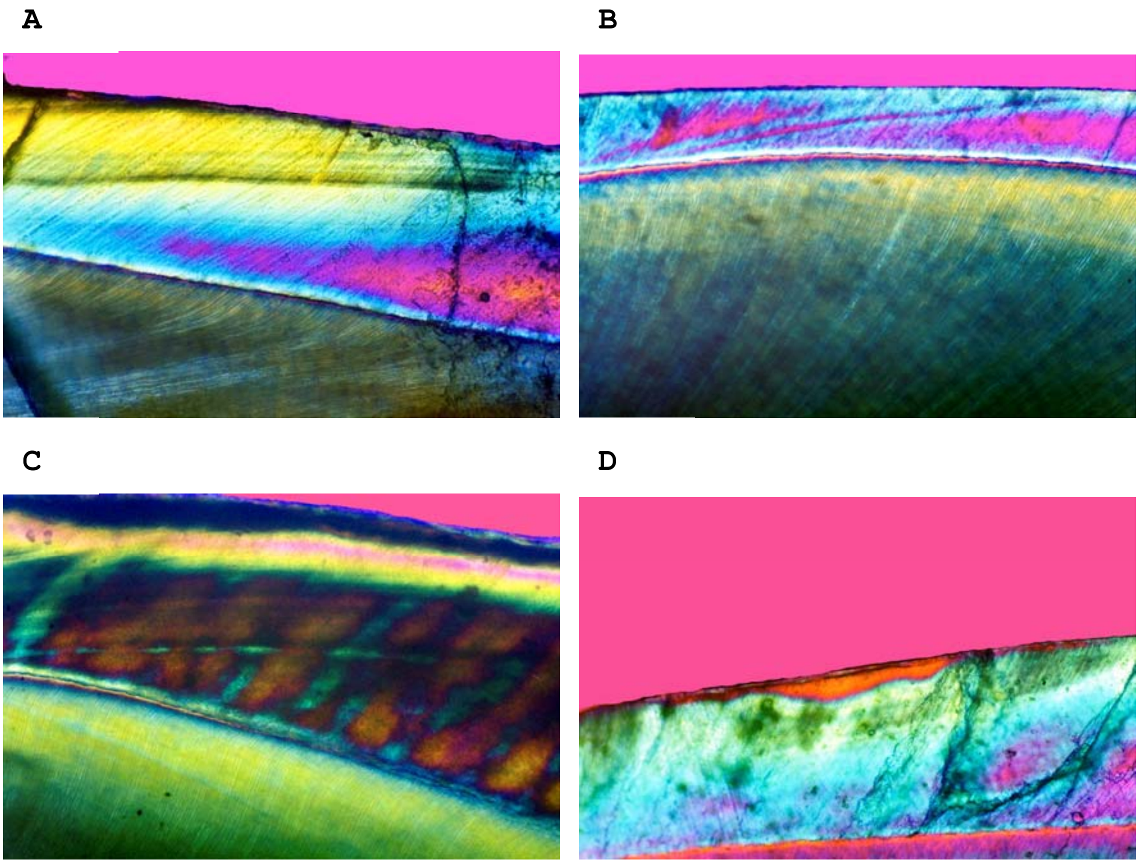


Figure 1A-1D

A B

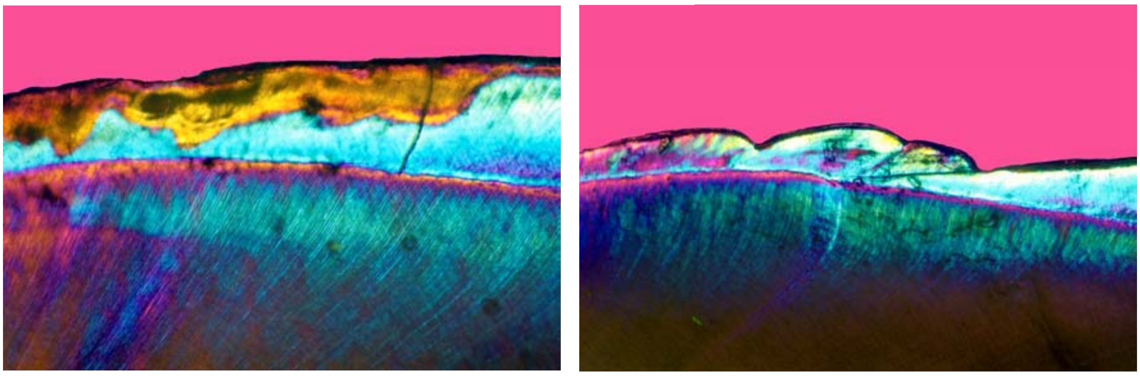


Figure 2A-2B

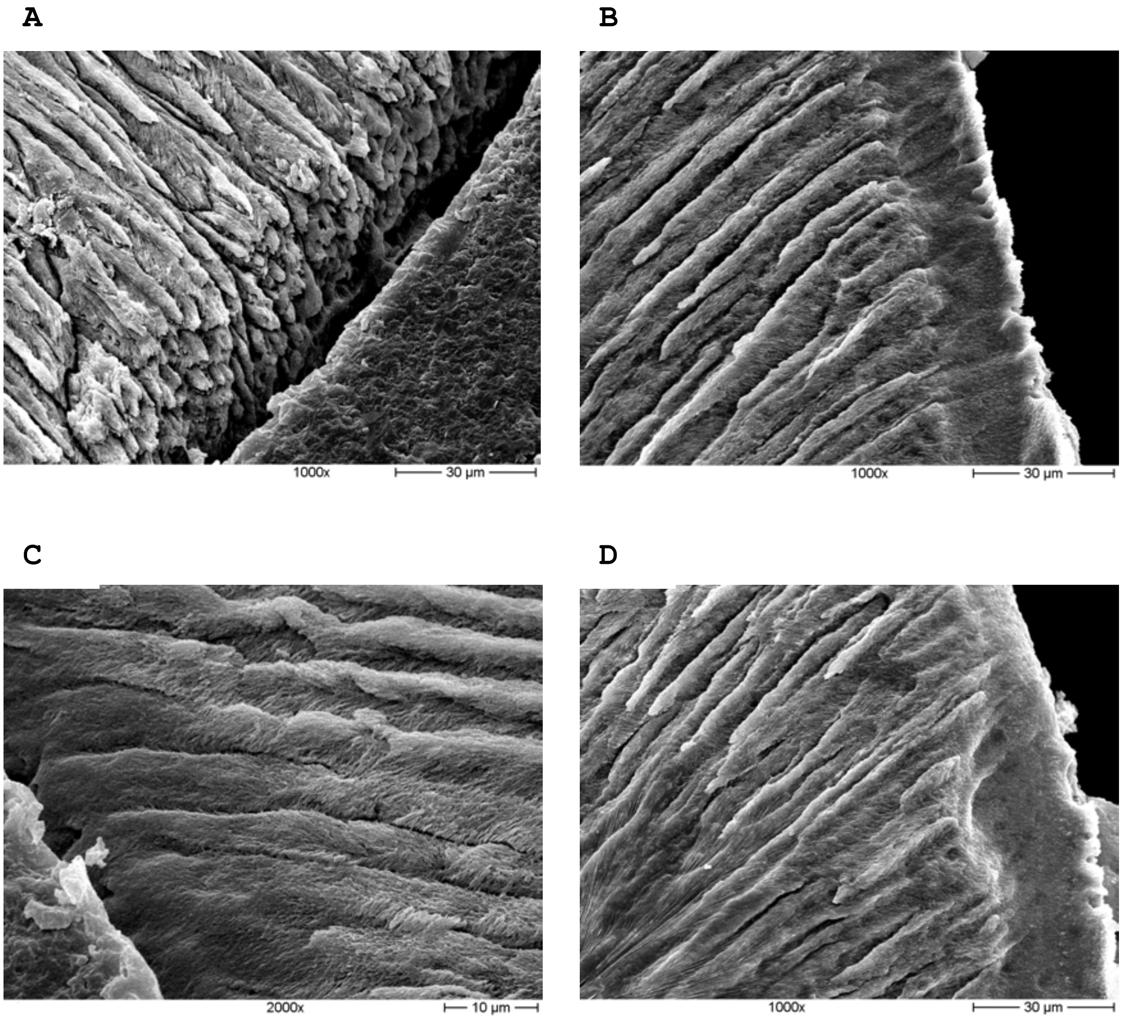


Figure 3A-3D