Salivary Lysozyme in Relation to Dental Caries among Thai Preschoolers

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Aim: *The objective of this study was to analyze salivary lysozyme levels and activities in Thai preschoolers with different dental caries status.* ***Study design:*** *Unstimulated saliva samples were collected from 64 preschoolers, divided into a caries free group (n = 32) and a severe early childhood caries (S-ECC) group (n = 32).* ***Results:*** *Both groups were similar regarding gender, age, dental caries status, salivary flow rate, and salivary protein concentrations. No differences were also in the caregivers´ characteristics, oral health behaviors, and feeding habits. Only professional fluoride application was less frequently found in the S-ECC group ($p < 0.03$). Western blotting and lysoplate assays revealed that salivary lysozyme levels and activities were significantly increased in the S-ECC group compared with the caries free group ($p< 0.001$; $p = 0.008$, respectively).* ***Conclusion:*** *The up-regulated expression of salivary lysozyme and the increased lysozyme activity in S-ECC preschoolers suggests a possible connection between salivary lysozyme and oral immunity in response to early childhood dental caries.*

Key words: *Dental caries, Lysozyme, Saliva, Preschoolers*

INTRODUCTION

Dental caries is the most common oral disease in children in many developing countries including Thailand. The 7th National Oral Health Survey in 2012 indicated that the dental caries rate was as high as 78.5 percent in Thai children aged 5 years.[1] The pathogenesis of dental caries is associated with various factors including teeth, the amount of fermentable carbohydrate, and acid-producing bacteria in the oral cavity. A complex interaction over time between these factors plays an essential role in caries development.[2] Importantly, the pathologic process of dental caries is modified by saliva. The significant role of saliva in prevention of dental caries is well documented because saliva is known to contain a large number of proteins with antimicrobial activity such as immunoglobulins, peroxidase, agglutinins, mucins, lactoferrin, and lysozyme.[3] These salivary proteins interact with cariogenic bacteria by various mechanisms such as prevention of bacterial aggregation and adherence, and inhibition of their metabolism and multiplication resulting in bacterial cell death.[3] Indeed, the importance of antimicrobial factors in saliva for dental caries is well established, but data on salivary lysozyme in relation to caries are rather scare, particularly, in children.

Lysozyme, a small molecule (approximately 14.7 kDa) with strong cationic property, can lyse bacterial cells by cleaving β (l-4)-glycosidic bonds between muramic acid and N-acetylglucosamine residues in the peptidoglycan of the bacterial cell wall.[4] It promotes degradation of bacterial cell walls through mechanisms such as activation of bacterial autolysins[5], bacterial aggregation[6], inhibition of bacterial adherence[7], and inhibition of bacterial metabolism.[8] Studies on the role of salivary lysozyme in dental caries susceptibility were mainly performed in stimulated saliva of adults and teenagers, but results are controversial.[9-15] Although no association between lysozyme activity and caries development was reported in some studies in adults[11,12,14], one report demonstrated lower levels of lysozyme in unstimulated saliva of the subjects with low caries increment.[14] Otherwise, studies in 12-year-old children did not reveal an association between salivary lysozyme activities and caries increment.[13,15]

Studies of salivary lysozyme in preschool children with dental caries are even more limited. One previous report showed

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significantly higher lysozyme activities in caries-free preschoolers compared with the caries-susceptible group.[9] However, a recent study in Chinese preschool children found no significant differences in levels of salivary lysozyme between the caries-free and caries susceptible groups.[10] So far, no studies have investigated the quantity and quality of salivary lysozyme in preschoolers with dental caries. We hypothesized that alteration of the quantity and quality of salivary lysozyme in the preschoolers might be related to dental caries susceptibility. It was the aim of this study to compare these parameters in a well characterized collection of preschoolers with severe dental caries and preschoolers without carious lesions.

MATERIALS AND METHOD

Study population

This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE500907). Written consent was given for each child from the caregiver. Sample size estimation was performed using Java applets for power and sample size[16], based on the data from Twetman.[9] Using this approach, we determined a study population of 64 preschoolers aged between 4 years and 5 years 11 months, from the "Demonstration School of Khon Kaen University", Khon Kaen University, Khon Kaen, Thailand. Thirty two preschoolers were selected for the severe-caries group and the other 32 preschoolers were selected for the caries free group. Inclusion criteria for the severe caries and caries free preschoolers included no apparent systemic diseases, no medication use, no intra-oral appliance use, and normal salivary flow rate. To ensure the caries-free status in the caries free preschoolers, bitewing dental radiographs with tight contact areas were taken after tooth brushing. Decayed, missing, and filled primary teeth (dmft) and surfaces (dmfs) were assessed according to the dental caries diagnostic criteria of the World Health Organization (WHO).[17] The children with dmft = 0 were selected into the caries free group, and the children with dmft ≥ 8 were selected into the severe early childhood caries (S-ECC) group.

A dental examination was performed by two dentists, using a mouth mirror and light at the school with a knee-to-knee approach. These examiners were trained for caries detection by examining 20 children aged 4-6 years. After training, a kappa index for inter-examiner consistency in caries assessment was analyzed. The value of kappa index was 0.94, indicating a high agreement level. Unstimulated saliva samples were collected between 09:00 and 11:00 am. The salivary flow rates were calculated immediately after the collection. Only samples from children having a normal flow rate (³ 0.3 ml/min) were selected for further analysis. Thereafter, saliva samples were centrifuged at 3000 rpm for 2 min at room temperature and the supernatant was stored frozen at –80 °C until analysis.

To assess whether any differences in salivary lysozyme levels could be ascribed, total protein concentrations were measured by the Lowry's method.[18] Thereafter, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) was used to separate salivary proteins as previously described.[19] Each saliva sample was prepared by mixing it with 2× solubilizing buffer (ratio 1:1). After boiling the mixture for 5 min, salivary proteins were separated by electrophoresis at 150 V. Molecular mass standards (Amersham Bioscience, Piscataway, NJ) phosphorylase b, 97 kDa; albumin, 66 kDa; ovalbumin, 45k Da; carbonic anhydase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α lactalbumin, 14.4 kDa were run on each gel. Separated proteins were stained with Coomassie brilliant blue R-250. The intensity (INT)/mm^2) of bands in the salivary samples at approximate 15 kDa (standard lysozyme 14.7 kDa) was determined on a BIO-RAD gel scanner, using the program Quality One (Bio-Rad, Hercules, CA)

Western blotting was performed to determine lysozyme levels in salvia.[20] After separating by electrophoresis, salivary proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA.) in transfer buffer containing 48 mM Trisbase, 39 mM glycine buffer containing 20% methanol at a constant current of 130 mA for 1 hour. Thereafter, the membrane was incubated in blocking solution [5% (w/v) skimmed milk in TBST buffer] at room temperature for 1 hour and then incubated with an 1:100, v/v dilution of a mouse-anti human lysozyme antibody (Abcam, Cambridge,) at room temperature for 1 hour. After washing the membrane 3 times for 5 min each with TBST [10 mM Tris-HCL, 150 mM NaCl containing 0.05% (v/v) Tween-20], the membrane was incubated with an alkaline phosphatase conjugated-goat anti mouse IgG (1:500, v/v in TBST) for 1 hour at room temperature. Then, the membrane was rinsed for 3 min 3 times with TBST, followed by TBS, pH 8.0 (10 mM Tris-HCl containing 150 mM NaCl) washing for 3 times, before the substrate buffer, pH 9.5 (100 mM Tris-base, 100 mM NaCl containing 50 mM $MgCl_2.6H_2O$) was applied twice for 30 sec each at room temperature. Visualization of immunoreactive bands was carried out by incubating the membrane in a substrate solution containing 30 µL *ρ*-nitroblue tetrazolium chloride (NBT) and 30 µL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 5 mL substrate buffer, pH 9.5 for at least 5 min. The color reaction was stopped by transferring the membrane into water. A control membrane with the molecular markers was stained with 0.1% amido black. The immunoreactive bands were quantified by measuring the intensity of bands, using the program Quality One (Bio-Rad).

The activity of salivary lysozyme was measured using the lysoplate method.[21] Human lysozyme was used as a standard (Calbiochem, San Diego, CA). Plates with 1% agarose gel supplemented with lyophilized *Micrococcus luteuse* cells. Ten µL of the saliva samples or standards (human lysozyme) were placed in the 5-mm wells and the lysoplates were incubated for 24 hours at 37 °C. Diameters of lysis zones were determined directly with a dial caliper (Mitutoya; Laboratory Supplies, Hicksville, NY) with accuracy to 0.02 mm. From the standard values plotted on a semilogarithmic graph, the enzymatic activity for each sample was calculated and normalized to the protein concentration (µg/mg protein).

Statistical analysis

Pearson's chi-square test was used to analyze categorical data. Shapiro-Wilk statistic was used to assess the normal distribution of continuous data. Mann-Whitney U test was utilized to assess statistical differences between S-ECC and caries free groups for skewed data, and independent t-test for normally-distributed data. Spearman's rank correlation coefficients were used to determine the correlations between salivary lysozyme and dental caries status. P-values <0.05 were considered statistically significant.

Table 1. Characteristics of the severe early childhood caries (S-ECC) and caries free groups by gender, age, dental caries status, salivary flow rate, and total protein concentrations

	S-ECC	Caries free	P-value
Gender [n (%)]			
Male	17 (53.1)	13 (40.6)	0.32 [a]
Female	15 (46.9)	19 (59.4)	
Age (month)			
Mean ± SD	57.8 ± 8.2	56.9 ± 7.2	0.50 [b]
dmft[€]			
Mean ± SD	12.5 ± 3.2	0	
dmfs[§]			
Mean ± SD	26.8 ± 15.5	0	
Salivary flow rate (ml/min)			
Mean ± SD	0.8 ± 0.4	0.8 ± 0.3	0.93 [b]
Total protein in saliva (mg/ml)			
Mean ± SD	1.2 ± 0.4	1.2 ± 0.3	0.98 [c]

[a] Chi-square test
[b] Mann-Whitney U test
[c] Independent sample t-test
[€] dmft: Decayed, missing, and filled primary teeth
[§] dmfs: Decayed, missing, and filled surfaces of primary teeth

Table 2. Characteristics of the severe early childhood caries (S-ECC) and caries free groups by caregiver´s characteristics, oral health behaviors, and feeding habits

	S-ECC n (%)	Caries free n (%)	P-value
Main care-giver			
Father	11 (34.4)	4 (12.5)	0.08[a]
Mother	21 (65.6)	27 (84.4)	
Grandparent	0 (0)	1 (3.1)	
Care-givers education level			
High school	6 (18.8)	3 (9.4)	0.36[a]
College	20 (62.5)	19 (59.4)	
University	6 (18.8)	10 (31.3)	
Tooth-brushing (time/day)			
1	3 (9.4)	0 (0)	0.21[a]
2	22 (68.8)	24 (75.0)	
≥3	7 (21.9)	8 (25.0)	
Brushing person			
Child	20(62.5)	21(65.6)	0.81[a]
Parent	8(25.0)	6(18.8)	
Child and parent	4(12.5)	5(15.6)	
Use of floss			
Never	26 (81.3)	22 (68.8)	0.25[a]
Irregular	6 (18.8)	10 (31.3)	
≥ once/day	0 (0)	0 (0)	
Professional fluoride application			
Never	16 (50.0)	11 (34.4)	**0.03[a]**
Irregular	14 (43.8)	11 (34.4)	
twice/year	2 (6.3)	10 (31.2)	
Systemic fluoride supplementation			
Never	29 (90.6)	26 (81.3)	0.43[a]
Irregular	3 (9.4)	5 (15.6)	
Regular	0 (0)	1 (3.1)	
Child's dental visit			
Never	7 (21.9)	7 (21.9)	0.21[a]
Only when having symptoms	12 (37.5)	6 (18.8)	
1-2 times/year	13 (40.6)	19 (59.4)	
Milk feeding behavior			
Bottle	7 (21.9)	3 (9.4)	0.17[a]
Straw or glass	25 (78.1)	29 (90.6)	
Milk flavor			
Plain	13 (48.4)	18 (56.3)	0.21[a]
Others	19 (59.4)	14 (43.8)	
Age at starting tooth brushing			
Mean ± SD	11.3 ± 6.8	9.5 ± 3.7	0.43[b]

[a] Chi-square test
[b] Mann-Whitney U test

RESULTS

The characteristics of the caries free and S-ECC groups were demonstrated in Tables 1 and 2. There were no significant differences in gender, age, salivary flow rate, and total salivary protein concentrations between the two groups. Because there are major concerns about the role of caregivers in the development of dental caries, differences in caregivers' characteristics were assessed by the standardized questionnaire. No significant differences in oral health behaviors, feeding habits, and fluoride substitution were observed between the two groups in which all caregivers had higher education (at least a high school level). Most parents of both groups allowed their children brushing by themselves twice a day and never used dental floss for their children. It should be noted that only half of children in the S-ECC group received professional fluoride application, whereas two thirds of children in the caries free group obtained this treatment. One third of the children in the latter group also had a regular professional fluoride application twice a year, whereas this treatment was done for 6% of the children in the S-ECC group. Supplementation with fluoride tablets or droplets did not make significant differences between these two groups.

Salivary lysozyme content and activity

In SDS-PAGE analyses, salivary protein bands with a molecular mass of approximate 15 kDa which is compatible to the molecular weight of human lysozyme were detected in each sample in both groups (Figure 1). We also checked for equal protein loading in the sample by measuring the density of an unrelated protein giving uniform bands at 30 kDa (Figure 1). Because there might be other salivary proteins with a molecular mass similar to that of lysozyme presenting at the protein band compatible to lysozyme, Western blotting was performed to quantify the lysozyme content in the samples. Indeed, we could demonstrate immunoreactive bands at 15 kDa corresponding to human lysozyme in both groups (Figure 2). Densitometric analysis revealed that the intensity of these lysozyme bands was approximately 30% higher in the S-ECC group as compared with the caries free group ($p < 0.001$) indicating the significantly higher content of lysozyme in this group (Table 3).

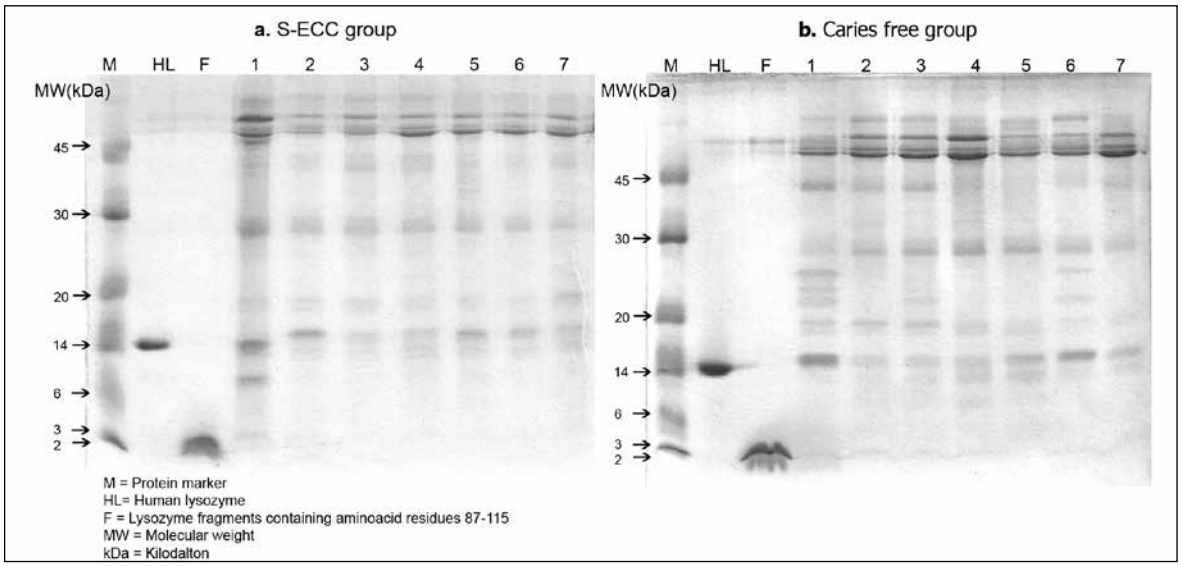
Lysoplate analyses demonstrated lysozyme activity is retained in the saliva collected from the two investigated groups. Lysozyme activity in the saliva of the S-ECC group was approximately 30% higher than that of the caries free group ($p = 0.008$, respectively) (Table 3). There were no significant correlations between salivary lysozyme content and dental caries parameters (dmft and dmfs).

Table 3. Comparison of the quantity (Western blot) and quality (lysoplate assay) of salivary lysozyme between the severe early childhood caries (S-ECC) and the caries free groups

	S-ECC Mean±SD	Caries free Mean±SD	P-value
Salivary lysozyme:			
Western blotting (INT/mm^2)	10,345.3 ± 2,337.6	6,781.9 ± 2,128.6	**< 0.001**[a]
Lysozyme activity (lysoplate assay)			
(µg/ml)	24.4 ± 15.2	16.3 ± 10.2	0.02[a]
(µg/mg protein)	20.2 ± 11.7	13.6 ± 6.8	**0.008**[a]

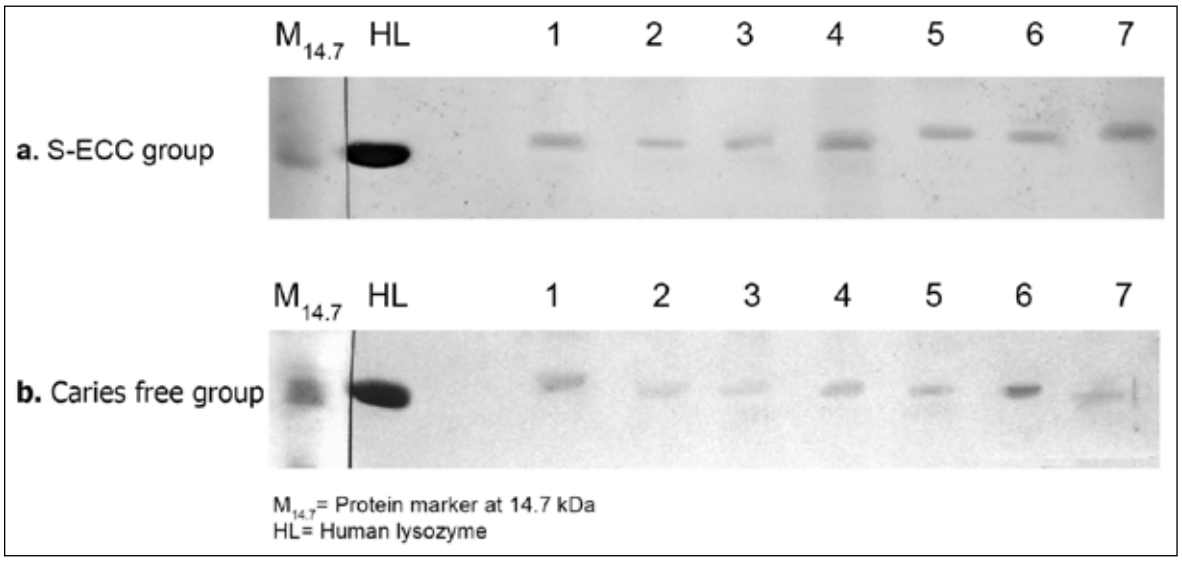
[a] Mann Whitney-U test
INT: Intensity (Arbitrary Units)

Figure 1. SDS-PAGE analyses of salivary (10 mg total protein/sample) in the caries free and the severe early childhood caries (S-ECC) groups. Protein bands which a molecular mass of approximate 15 kDa similar to that of human lysozyme (14.7 kDa) suggest presence of lysozyme in all samples. A uniform intensity of protein bands at a higher molecular mass indicates uniform sample loading.



Note that protein bands have a higher intensity intense in the caries free group (see Table 3 for quantitative evaluation).

Figure 2. Western blotting analyses of salivary samples demonstrate presence of lysozyme in all samples. Immunoreactive bands, representing lysozyme, are more intense in samples from the severe early childhood caries (S-ECC) group than in the caries free group.



DISCUSSION

The present study compared the quantity and quality of salivary lysozyme between the S-ECC group consisting of preschoolers with severe dental caries and the controls without dental caries. According to our Western blot and lysoplate assay data, increased levels and the corresponding activity of salivary lysozyme are seen in the S-ECC group as compared to the controls. Our findings are consistent with data from a 4-year study in young adults, in which lower lysozyme levels were seen in unstimulated saliva of volunteers with low caries increment.[14] However, the data are contrary to another report revealing no significant differences in the lysozyme concentrations between the caries-susceptible and the caries-free preschool children.[10] Regarding lysozyme activities, our findings are in good agreement with one previous study.[22] On the other hand, our lysoplate data in unstimulated saliva of the S-ECC group are in contrast to another study reporting lower lysozyme activities in stimulated saliva of caries-susceptible preschoolers.[9] Our lysozyme activity data are also in contrast to other studies which found no significant differences in the lysozyme activities in stimulated saliva among subjects with different caries status.[12-14]

Although increased levels of salivary lysozyme and increased lysozyme activities in the groups of preschoolers with severe caries were observed in our study, no significant correlations between salivary lysozyme content and dental caries status were demonstrated. It should be noted that in preschool children aged between 4-6 years in our study represented an oral environment with deciduous dentition, whereas young adults in the previous study[14] represented an oral environment with permanent dentition. Therefore, it would be hypothesized that shift in tooth development from deciduous to permanent dentition might affect the patterns of correlations between expression of salivary lysozyme and lysozyme activities in response to dental caries. However, inconsistent results between previous and present studies may be due to different variables in the experimental conditions which could affect the salivary lysozyme content and activity, e.g. differences in the types of collected saliva, oral environments with different tooth dentitions, or aging. Other factors such as criteria for establishing dental caries status, and different methods for measuring salivary lysozyme may result in different outcomes. Therefore, standardization of methods for measurement of salivary lysozyme at various conditions of oral environments would be of importance to validate the role of salivary lysozyme in the development of early childhood caries.

CONCLUSION

The present study demonstrated the increased levels of salivary lysozyme together with increased lysozyme activities in preschoolers with severe dental caries as compared with the caries free group. These findings suggest a possible connection between salivary lysozyme and oral immunity in response to early childhood dental caries. However, intensive studies with standardized methods and larger sample size are needed to confirm the role of salivary lysozyme in pathogenesis of early childhood dental caries.

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