Risk factor of secretory immunoglobulin A and salivary lysozyme level in children aged under 3 years to severe early childhood caries

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Abstract. Severe Early Childhood Caries (SECC) is a significant dental health problem in various countries. The caries pathology process is affected by saliva status. An important antimicrobial component in saliva is slgA preventing the attachment of bacteria to enamel and lysozyme capability to act as an antibacterial by hydrolyzing bacterial polysaccharide walls. The aim of the study is to compare the slgA level and salivary lysozyme between children with caries free and suffering from SECC, aged under 3 years. This study is an observational analytic with cross sectional design. Saliva samples were collected unstimulated from 68 children. Examination of slgA level and salivary lysozyme used ELISA sandwich technique. Data were analysed using Mann-Whitney and Kruskal Wallis, while correlation test used Spearman. The results showed, there was no significant difference in slgA level and salivary lysozyme between caries-free children and SECC children (p > 0.05). It has been found that in caries-free children have a higher mean lysozyme level than SECC children. It has been found a medium correlation between deft and lysozyme level (r = -0.492). In conclusion, lysozyme level was a risk factor for the incidence of SECC in children aged under 3 years. The higher deft status, the lower lysozyme level of the child.

1. Introduction

Early Childhood Caries (ECC) according to the American Academy of Pediatric Dentistry (AAPD), is the presence of carious lesions on the surface of primary teeth (cavities or non-cavities), the tooth lost due to caries or filled at children aged under 6 years old. Severe Early Childhood Caries (SECC) is more severe form of caries than ECC. The definition of SECC are: a) the presence of caries on one smooth surface of the teeth of children under 3 years old, b) the presence of caries, teeth lost due to caries or filled on the smooth surface of the primary anterior maxillary teeth of children aged 3-5 years, c) children have the deficit index is greater than or equal to 4 in children aged 3 years, greater than 5 or same as in children aged 4 years, and greater or equal to 6 for children aged 5 years [1].
ECC and SECC are significant dental health problems throughout the world. The prevalence of ECC and SECC is still high and even increasing in various countries, especially in developing countries [2-6]. ECC prevalence in Xianjiang City (China) was found 78.2%, while SECC was 41.2% [3]. ECC prevalence in Vietnam was found to be very high at 91.9 % [4]. Research in Northern Thailand revealed SECC prevalence at 44.1% [5]. In Medan City (Indonesia) ECC and SECC prevalence was found in children aged under 3 years at 57.7% and 16% [6].

The pathogenesis of caries is associated with major factors such as teeth, fermented carbohydrates, and production of acid by bacteria in the oral cavity. Interaction between time and those three factors play a role in caries development [7]. One important thing to know is that caries pathology process is influenced by saliva [8]. The role of saliva as caries prevention has been widely reported. This is related to saliva containing a large number of antibacterial proteins such as immunoglobulins, lysozyme, peroxidase, lactoferon, agglutinin, mucin, defensin, histatin [9].

An important antimicrobial component in saliva is secretory IgA (slgA). slgA serves to prevent attachment of cariogenic bacteria to enamel [10]. Pal et al. reported that the highest slgA total number are caries-free children, followed by groups of children with moderate caries (1-4 teeth) and the lowest is the group of children with high caries (5-15 teeth) [11]. Different results reported that no difference of total slgA level between caries-free children with children having ≥ 5 deft [12].

Lysozyme acts as an antibacterial, antivirus, and antitumor and immune modulator activity. Lysozyme has muramidase activity, which is able to hydrolyze β(1-4) bonds between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of bacterial cell walls, so that it can hydrolyze bacterial polysaccharide walls in various situations such as hypo-osmolarisation [13]. Researchers reported that lysozyme level in caries-free children was higher than ECC children (p=0,04) [13]. Different results are reported that there was no difference in lysozyme level between caries-free children and children with caries (df>5) [14].

Considering inconsistent results found between the protective mechanics of saliva, especially the slgA protein and lysozyme in incidence of caries, therefore further research is needed. Until now, research on the quantity of slgA and lysozyme in children aged under 3 years is still few. If we can find out the risk factors of caries in younger children, we can prevent caries incidence earlier. Therefore, the aim of this study is to compare the slgA level and salivary lysozyme between groups of children aged under 3 years who are caries-free and suffering from SECC.

2. Materials and methods

2.1. Subjects
The research is analytic observational with cross-sectional design. The sample size of this study is 68 children aged under 3 years, consisting of 34 caries-free children and 34 SECC children. The subjects were obtained from Posyandu at Puskesmas in Medan Selayang, Medan Sunggal and Medan Johor by purposive sampling.

Criteria of the subjects are having at least 2 primary upper incisors tooth which had erupted, a good general state, not taking medications which can affect the salivary at least 1 month prior to the study, parents to fill out an informed consent. Criteria for SECC children based on the American Association of Pediatric Dentistry (AAPD) is children suffering from caries which minimum 1 incisor tooth on a smooth surface or 1 filled tooth on a smooth surface or 1 extracted tooth due to caries. Caries-free child defined as not having dental caries or filled teeth or extracted teeth due to caries. This research has been approved by the Ethics Committee of Faculty of Medicine University Sumatera Utara.

2.2. Caries examinations and salivary sampling
All children had their teeth examined visually by sunlight. Caries examination used a mouth mirror and half moon explorer. Caries examination results were recorded in a questionnaire.
2.3. Saliva chemical examination
The chemical examination that would be carried out is the examination of sIgA level and lysozyme in saliva.

a. Examination of the sIgA level with sandwich technique using the Human sIgA ELISA Kit (Fine Test, H3778D028). The work techniques are:
   1. Saliva samples were stored at -20°C, if the sample would be used, it must be placed at room temperature until molten (about 2 hours). Microtube saliva was centrifuged with a centrifugal machine for 1000 spins / minute for 20 minutes at 4°C.
   2. Sample Preparation
   The sample was diluted 100 times by 1 μl saliva added by 99 μl buffer fluid. In this study, sample dilution of saliva was varied, ie 100,200,300 and 400 times, depending on the concentration of sIgA owned by the respective saliva subject (if dilution was 100 times unreadable, then dilution would be increased).
   3. Reagent preparation according to manufacturer's procedure such as wash buffer, standard, and biotin-labeled antibody, and HRP-Streptavidin Conjugate (SABC). Standard without dilution serves as a high standard (200 ng / ml) and diluent arrays (buffer solution) as standard zero (0 ng / ml), so the standard used was 200 ng / ml, 100 ng / ml, 50 ng / ml, 25 ng / ml, 12.5 ng / ml, 6.25 ng / ml, 3.12 ng / ml and 0 ng / ml.
   4. Micro plate (well) was previously washed twice with automatic washing machine.
   5. Additional 100 μl of standard solution and sample on each well, then well was closed with adhesive strip. The plate then be sealed with a cover and incubate at 37°C for 90 minutes.
   6. Then well is coated and washed twice. Next 0.1 ml of biotin-labeled antibody was added on each well, then well was covered with a new adhesive strip and incubated for 60 min at 37°C.
   7. Then adhesive strip was removed, and the plate content was discarded, and plate was washed 3 times with wash buffer, and well was soaked for 1 minute using wash buffer for each time. The plate was turned and the liquid was removed on tissue paper. Then 0.1 ml SABC was added on each well, then sealed with adhesive strip and incubate for 30 min at 37°C.
   8. The aspiration and the washing process was repeated for 5 times as in step 7. Next, 90μl TMB substrate was added on each well (light avoided). Incubation was carried out for 15 minutes at 37°C.
   9. 50μl stop solution was added on each well, carefully the plate was tapped to ensure the solution mixed. Then reading of the microplate reader was immediately done. Optical density was determined using a microplate reader with absorbance at 450 nm.

b. Examination of lysozyme level with sandwich technique using the Human LZMc ELISA Kit (Fine Test, H4052D028). The work techniques are:
   1. Sample Preparation
   In this study, dilution of salivary samples was carried out variably at 10 and 100 times, depending on the lysozyme concentration of each subject saliva (if dilution is 10 times unreadable, then dilution is increased).
   2. Reagent preparation according to manufacturer's instructions such as wash buffer, standard, and biotin-labeled antibody, and HRP-Streptavidin Conjugate (SABC). Then well was washed twice. Then 50 μl standard and sample were added in the well, after which 50 μl of Biotin-detection antigen was added in each well, incubated for 45 minutes at 37°C.
   3. Well aspiration and washing was done 3 times, then 100 mL of SABC was added on each well, then incubated for 30 min at 37°C. Then aspiration and washing was done 5 times.
4. 90 µl TMB was added on each well, then incubated for 10 minutes. After that 50 µl stop solution was added on each well and was read immediately with wavelength 450 nm.

2.4. Data analysis
Analysis test to determine the mean difference of sIgA level and lysozyme between caries-free children and SECC (2 groups) used Mann-Whitney test because the data is not normally distributed. The Kruskal Wallis test was used to analyze the mean difference of sIgA level and lysozyme among 4 groups of children according to the category of deft because the data is not normally distributed. Correlation test between deft and lysozyme level was tested using Spearman, because the data is also not normally distributed. The significance value used is $p < 0.05$.

3. Results

3.1. Subject characteristics
The characteristic features of the research subjects in two groups of caries-free children and SECC children are each composed of 18 males (52.94%) and 16 females (47.06%). The mean age for children with caries-free group is $16.24 \pm 4.63$ months, while SECC children group is $19.79 \pm 5.25$ months (Table 1).

The mean number of teeth owned by caries-free children is $10.26 \pm 4.74$, while the SECC children group is $13.32 \pm 4.64$. Based on deft, the mean deft in the SECC group is $3.97 \pm 1.99$. Based on the mean number of carious teeth compared with the mean number of teeth owned by children, it is obtained in the SECC group that the caries mean percentage of carious teeth is $30.95 \pm 15.52\%$ of the oral cavity, while healthy teeth is $69.05 \pm 15.52\%$ (Table 1).

| Variable                        | Caries Free (n = 34) | SECC (n = 34) | p  |
|---------------------------------|---------------------|---------------|----|
| Gender                          | Male                | 18 (52.94%)   | 18 (52.94%) |    |
|                                 | Female              | 16 (47.06%)   | 16 (47.06%) |    |
| Mean age of child (month)       | 16.24 ± 4.63        | 19.79 ± 5.25  |    |
| Mean number of children's teeth | 10.26 ± 4.74        | 13.32 ± 4.64  |    |
| Mean deft                       | 0                   | 3.97 ± 1.99   |    |
| Mean number of healthy teeth (%)| 100                 | 69.05 ± 15.52 |    |
| Mean number of carious teeth (%)| 0                   | 30.95 ± 15.52 |    |

3.2. Comparison between sIgA level and lysozyme in caries-free children and SECC
The result shows no mean difference of sIgA level between caries-free children and SECC ($p = 0.277$). On the contrary, a statistically significant mean difference ($p = 0.001$) of lysozyme level was found between groups of caries-free children ($3142.47 \pm 2554.86$ ng / ml) and SECC children ($1169.06 \pm 1459.29$ ng / ml). Caries-free children have higher mean lysozyme level than SECC children (Table 2).
Table 2. Mean comparison of sIgA level and lysozyme among group of caries free and SECC children

| Child Category | n  | Mean sIgA level (ng / ml) | p   | Mean lysozyme level (ng / ml) | p    |
|----------------|----|-------------------------|-----|-------------------------------|------|
| Caries free    | 34 | 702.09 ± 420.82         | 0.227| 3142.47 ± 2554.86             | 0.001|
| SECC           | 3  | 601.50 ± 424.23         |      | 1169.06 ± 1459.29             |      |

3.3. sIgA and lysozyme level comparison based on caries category

Based on the category of child caries, a high mean sIgA level was found in the caries-free group (702.09 ± 420.82 ng / ml) and low caries group (714.86 ± 406.86 ng / ml) compared to medium caries groups (527.58 ± 460.52 ng / ml) or high caries group (514 ± 404.87 ng / ml), but the mean difference is not statistically significant (p = 0.209) (Table 3).

There is a difference of lysozyme level between groups of caries-free children and groups of children with low, moderate and high caries categories (p = 0.001) (Table 3). From the post Hoc results using the Mann-Whitney test on the examination of lysozyme level based on caries category, it was found a group of children with different mean lysozyme level were between caries-free group (3142.47 ± 2554.86 ng / ml) with low caries categorical group (1213, 79 ± 1806,511 ng / ml; p = 0,002) and the caries-free group with medium caries group (940,68 ± 1074,75 ng / ml; p = 0,001), while the other groups did not show any significant differences.

Table 3. Mean comparison of sIgA level and lysozyme based on caries category

| Caries Category | n   | Mean sIgA level (ng / ml) | p    | Mean Lysozyme level (ng / ml) | p    |
|-----------------|-----|-------------------------|------|-------------------------------|------|
| Caries free     | 34  | 702.09 ± 420.82         | 0.227| 3142.47 ± 2554.86             | 0.001|
| Low caries (deft = 1-3) | 14  | 714.86 ± 406.86         | 0.209| 1213,79 ± 1806,511            | 0.001|
| Medium caries (deft = 4-5) | 12  | 527,58 ± 460,52         | 940,68 ± 1074,75                      |      |
| High caries (deft = 6-8)    | 8   | 514 ± 404,87            | 1433,38 ± 1410,71                      |      |

Post hoc results with the Man-Whitney testobtained: a: b = 0.002 and a: c = 0.001

3.4. Correlation between deft and lysozyme level

Correlation was obtained between deft and the lysozyme level (p = 0.001). The Spearman correlation value obtained is -0.492, and this correlation has moderate strength. The direction of correlation is negative, which means the higher deft, the lower lysozyme level (Table 4).

Table 4. Correlation between deft and lysozyme level

| Number of children (N) | Mean deft | Mean Lysozyme Level (ng /ml) | p    | r    |
|------------------------|-----------|-------------------------------|------|------|
| 64                     | 1.99 ± 2.44| 2155.77 ± 2291.71             | 0.001| -0.429|
4. Discussion
This study was carried out on very young children which aged under 3 years. The percentage of teeth involved in caries is around 30.95% with def of 3.97 ± 1.99, while the mean number of teeth which had erupted in this child was 13.32 tooth (Table 1). This is wistful enough, since a young child with a number of erupted teeth is still 13 tooth, but 30% of them have been involved in caries. The attention of health personnel is needed to detect early caries in very young children. Caries prevention strategies must begin with education in pregnant women, then in the perinatal period and continued on mother and baby [2]. Parents are advised to maintain the health of child's oral cavity since the child is born. Parents are educated about the etiology of caries incidence and how to prevent them [15].

Salivary immune response to Streptococcus mutans (S. mutans) show individual characteristics in childhood. Children respond differently to S.mutans infection, this condition is the result of an expansion of infection (dose of antigen) or age at the time of infection (maturity of the immune response) [16]. Antibodies against S. mutans can be detected in saliva, but their relationship with the immune to caries is still contradictory [17].

The results of this study found no mean difference of slgA level between caries-free and SECC children (p>0.05), although substantially carious-free children have higher slgA level (702.09 ± 420.82 ng / ml) than SECC children (601.50 ± 424.23 ng / ml) (Table 2). The highest slgA level was found in carious-free children and slgA level decreased following caries increase (Table 3).

The results of this study are in accordance with Shifa et al [12] and Koga et.al [18] who also found no different slgA level between caries-free children and children with active caries. But the results of this study are different from those of Pal et al. who reported the highest total number of caries-free children, followed by groups of children who had moderate caries (1-4 teeth) and the lowest group of children with high caries (5-15 teeth) [11].

Lysozyme enzymes have small concentration in the salivary glands, but significant biological level as antibacterial [19]. The results of this study found caries-free children have higher mean lysozyme level (3142.47 ± 2 554.86 ng / ml) than SECC children (1169.06 ± 1459.29 ng / ml) (p <0.05) (Table 2). This result is in accordance with Moslemi et.al study [13].But the results of the study do not match with Lertsirivorakul et.al. [20] who found that lysozyme level was higher in SECC children than caries-free children (p <0.05). The high concentration of lysozyme in the active caries versus caries free groups may be due to the compensation mechanism. The presence of caries will increase the number of S. mutans, so that lysozyme as a defense mechanism will increase its secretion due to stimulation of the bacteria [21].

This study found that the highest lysozyme level belongs to a group of caries-free children, followed by a group of children with a low caries category (deft 1-3) and medium caries category (4-5 deft) (p <0.05) (Table 3). However, in the category of children with high caries, the mean number of lysozyme level is higher than in the category of children with low and moderate caries. This is possible because the number of study subjects in the high caries category is less than the number of subjects in the low and medium caries category. The results of this study are in accordance with the research of Musa et.al who found that children with low caries category had the highest lysozyme level followed by a group of children with moderate and severe categories.[22].

Correlation with moderate strength was found between deft and the lysozyme level (p = 0.001, r = -429). The higher deft, the lower children's lysozyme level (Table 4). The lysozyme level in saliva may be developed as a biomarker for detecting dental caries in early childhood. Future studies can be developed with longitudinal research designs to explain the antibacterial role of saliva in ECC and SECC events [17].

5. Conclusion
Lysozyme level was a risk factor for the incidence of SECC in children aged under 3 years. The higher deft, the lower children’s lysozyme level.
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References

[1] American Academy of Pediatric Dentistry (AAPD) (2014). Policy on early childhood caries (ECC): Classifications, consequences, and preventive strategies. Refer Man; 37: 50-52.

[2] Anil S, Anand PS (2017). Early childhood caries: Prevalence, risk factors, and prevention. Frontiers in Pediatrics; 5 (157): 1-7.

[3] Li Y, Wulaerhan J, Liu Y, Abudureyimu A, Zhao J (2017). Prevalence of severe early childhood caries and associated socioeconomic and behavioral factors in Xinjiang, China: A cross sectional study. Zhonghua Kou Qieng Yi Xue Za Zhi; 70: 17-144.

[4] Huong DM, Hang LTT, Ngoc VTN, Anh LQ, Son LH, Chu DT, Le DH (2017). Prevalence of early childhood caries and its related risk factors in preschoolers: Result from a cross sectional study in Vietnam. Pediatric Dent J; 30: 1-6.

[5] Peltzer K, Mongkolchati A (2015). Severe early childhood caries and social determinants in three-year-old children from Northern Thailand: A birth cohort study. BMC Oral Health; 15: 108.

[6] Octiara EA, Tamba EA (2012). Hubungan ekonomi keluarga dan pendidikan ibu dengan Early Childhood Caries (ECC) anak usia 12-36 bulan di Kecamatan Medan Denai. Dentika Dent J; 17: 79.

[7] Newbrun E (1989). Current concepts of caries etiology. Textbook of cariology. Third Edition. Chicago: Quintessence Publishing Co; 29-44.

[8] Jayaraj D, Ganesan S. Salivary pH and buffering capacity as risk markers for early childhood caries: A clinical study. JCDP 2015; 26: 158-61.

[9] Muchandi S, Walimbe V, Jhawar G, Ito CY, Martins CA, Balducci I, Jorge AO (2014). Prevention and management of dental decay in the preschool child. Aust Dent J; 51(3): 272-5.

[10] Smith DJ (2002). Dental caries vaccines: prospects and concern. Crit Rev Oral Biol Med; 13: 335-49.

[11] Octiara EA, Sutadi H, Siregar Y, Primasari A (2017). slgA and lysozyme as biomarker of early childhood caries risk. Advances in Health Science Research; 8: 96-101.

[12] Koga-Ito CY, Martins CA, Balducci I, Jorge AO (2004). Correlation among mutants streptococci counts, dental caries, and IgA to Streptococcus mutans in saliva. Braz Oral Res; 18(4): 350-5.
[19] Amerongen AVN, Bolscher JGM, Veerman ECI (2004). Salivary protein: protective and diagnostic value in cariology?. Caries Res; 38: 247-53.

[20] Lertsirivorakul J, Petsongkram B, Chaiyarit P, Klayanongsruang S, Pitiphat W (2015). Salivary lysozyme in relation to dental caries among Thai preschoolers. Pediatr Dent; 39(4): 343-7.

[21] Leone CW, Oppenheim FG (2001). Physical and chemical aspects of saliva as indicators of risk for dental caries in humans. J Dent Educ; 65(10):1054-62.

[22] Musa HT, Zwain AM, Al-Mizraqchi AS (2018). Salivary lysozyme in relation to mutans streptococci among children with different stages of early childhood caries. http://www.researchgate.net/publication/327633889(Akses 15 Oktober 2018).