



The sIgA and histatin-5 expressions in saliva of early childhood caries and caries-free children based on period times

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ABSTRACT: To analyze the level of sIgA and Histatin-5 expression in Early Childhood Caries (ECC) and caries-free children. Saliva was collected from 31 subjects of ECC and 29 caries-free (ages 9-32 months). The level expression of sIgA and Histatin 5 was analyzed by enzyme-linked immunosorbent assay. The distribution and frequency of subjects consist of boys 47% and girls 53% with the average of ages 1-12 month 21% and 13-24 month 79%. sIgA and Histatin 5 expressions differ from the month periods tested (1, 3, and 9 months) but do not show any significant difference from each examination period (1 with three months; $p > 0.05$: 0.116), (1 with nine months; $p > 0.05$: 0.766), and (3 to 9 months; $p > 0.05$: 0.398). The expression of sIgA and Histatin 5 of boys and girls were not significant ($p > 0.05$: 0.550) and weak correlation ($r = 0.079$). The level expression of sIgA in the Caries-free higher than ECC ($p < 0.05$). Whereas the expression of Histatin 5 in ECC higher than Caries-free ($p < 0.05$). The sIgA correlating in ECC and caries-free is significant ($p < 0.05$) and no correlation ($r = -0.29$). Whereas Histatin 5 in the ECC and caries-free were strong correlation = 0.84. Both the result of ECC and caries-free suggested that the antibodies have been involved in the caries pathogenesis. In the ECC cases that occurred to decrease sIgA levels and increased of Histatin 5 levels, both antibodies have expressed in Caries-free Children. Both sIgA and Histatin 5 involved in the pathogenesis caries infection in children.

KEYWORDS: Antibodies; Early Childhood Caries; saliva; secretory immunoglobulin A; Histatin-5

INTRODUCTION

Early childhood caries (ECC) are a type of caries affecting infants and preschoolers, influencing children's development and health with the long-term effects caused. Early childhood caries is an essential consideration for all parties in reducing the risk of development and its causes, considering the infection process due to ECC can be prevented and is reversible.¹ Previous research concluded that ECC was due to *S. mutans* bacterial infection and host and dietary factors. Therefore, current research is trying to identify risk factors such as natural defenses in the oral cavity that can prevent caries development.²

Saliva is a body defense system containing various proteins, peptides, and immunoglobulin that prevent caries.³ Secretory immunoglobulin A (sIgA) is the dominant immunoglobulin in saliva that plays a vital role as the frontline defense of the

immune system against microbial invasion.⁴ sIgA is the main immunoglobulin of the mucosal immune system that acts as an antibody against bacterial antigens and inhibit the infecting bacteria of the oral tissue. The sIgA level reflects the condition, ability, or response of a person's body to form antibodies and describe the exposure of pathogens that mainly enter the oral cavity.⁵ Examination of sIgA levels in children with ECC needs to determine the child's adaptive immune response to exposure to *S. mutans*, a bacterium that causes caries.⁶

Reduction in the number of peptides in saliva in patients with active caries is associated with a person's susceptibility to caries.⁷ One of the salivary peptides that have antibacterial properties is Histatin-5. Statistical analysis shows a significant increase in Histatin-5 concentration in children with

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ECC.³ It indicated that the concentration changing of Histatin-5 during caries development has the potential to be an essential value to treat caries.⁸ However, until now, how is the relationship between sIgA and Histatin-5 to caries susceptibility

individually is not widely known. This study aims to evaluate sIgA and Histatin-5 expression in the salivary of ECC and Caries-free Children. These results would help predict the risk of early caries and strategies to prevent caries infection in children.

MATERIALS AND METHODS

The research was approved ethical clearance by Medical Faculty, Universitas Sumatera Utara, Medan Indonesia. Issued No. 41/TGL/KEPK FK USU-RSUP HAM/2018. The study was conducted for nine months in 2018 with the design of the Pre-test-Post test Control Group. The population consists of boys and girls from Medan and Banda Aceh (ages 9-32 months). Before the saliva collection from children, the subject's parents would sign an *informed consent*, and a dental examination was performed to determine the grouping of study subjects according to ECC's inclusion criteria and caries-free.

Sample Collection

Saliva collection of subjects was carried out with permission from their parents. The examination is carried out 1 hour after breakfast (around 08.00-11.00). The subjects' caries were examined by applying caries detector liquid on the surface of tooth enamel using cotton pellets. Furthermore, the liquid is cleaned using sterile cotton, and the results are recorded. An amount 2 mL aliquot of each unstimulated whole saliva was taken using a disposable pipette and filled in the disposable tube, marked, stored in an iced thermos to prevent protein hydrolysis, then was immediately taken to the laboratory (less than 2 hours after collection). The saliva collected for three-time repeats assays with the other month started by month 1 (January), 3 (March), and 9 (September) in 2018. The subjects' saliva was centrifuged to extract the sIgA (1000 rpm 20 min) and collect the Histatin 5 (10,000 rpm, 15 min), then stored at -20°C until the examination.

ELISA Assay of sIgA and Histatin 5

Sample and reagent preparations of Histatin-5 analysis was adopted by Elisa Kits of Wuhan Abebio Science Co., Ltd and sIgA analysis based on principled by Wuhan FineBiotechCo., Ltd. Examination procedure of Histatin 5, a total of 100µl standard and samples were put into each *well*, covered with an available adhesive film cover and incubated for 2 hours at 37°C. After 2 hours, each well was aspirated and washed with 250µl of an auto washer three times using and patted on a tissue. Then 100µl of biotin conjugate was added to each well, covered with an adhesive film coating,

and incubate for one hour at 37°C. Each well was aspirated and washed with 250 µl 3 times after an hour using an auto washer and patted on a tissue. Add 100µl of streptavidin-HRP to each well, cover with adhesive film cover, and incubate for one hour at 37°C. After an hour, each well aspirated and washed with 250µl 5 times with auto washer and patted on a tissue. Next, 100µl substrate solution was added into each well, covered with aluminum foil (to avoid light), and incubate for 15-20 minutes at 37°C. After 15-20 minutes, add 50µl stop solution to each well, and the color turned yellow. The values of sIgA expression read by ELISA reader at 450nm.

Examination procedure of sIgA: before pouring standard solutions and samples, the microplate was washed twice. A total of 0.1 ml of the standard and samples were poured into each well, covered with an available adhesive film cover, and incubated for one and a half hours at 37 °C. After one and a half hours, each well aspirated, washed, and soaked twice using an auto washer and patted on a tissue. Then, added 0.1 ml of biotin-labelled antibody working solution to each well, covered with adhesive film cover, and incubated for 1 hour at 37°C. After an hour, each well aspirated, washed, and soaked three times using an auto washer and patted on a tissue. Next, I added 0.1 ml of SABC working solution to each well, cover with an adhesive film cover, and incubated for 30 minutes at 37°C. After 30 minutes, each well was aspirated, washed, and soaked five times using the auto washer and patted on a tissue. Next step, added 90µl TMB substrate to each well, covered with aluminium foil (to avoid light), and incubate for 15-30 minutes at 37°C. After 15-30 minutes, added 50 µl stop solution to each well, and the color turned yellow and read by the ELISA reader at 450 nm.

Statistical Analysis

The level expression of sIgA and Histatin 5 of ECC and Caries-Free analyzed by Mann Whitney Test. In contrast, the period times expression of sIgA and Histatin 5 used in the Independent sample t-test and the total protein expressions of both analyzed by the Kruskal-Wallis test. The significant is $p < 0.05$ and strong correlation ($r = 1$).

RESULTS

The subjects' distribution and frequency consist of boys 47% and girls 53% with an average of ages 1-12 months 21% and 13-24 months 79%. Statistically, the expression of between sIgA and Histatin-5 of boys and girls groups was not significant ($p > 0.05$; 0.904 $r = -0.039$) and no

correlation. It has been suggested the boys and girls groups have the same opportunity to express the sIgA and Histatin-5 in saliva three times evaluated. The distribution of characteristic frequency and social subject data shown in **Table 1**.

Table 1. The Characteristic of distribution and social data of the subjects

Variables	n	%
Sex		
Boy	28	46,7
Girl	32	53,3
Ages		
1 - 12 month	13	21,7
13 - 24 month	47	78,3
Number of Children in the Family		
3 or more children	16	26,7
2 children	20	33,3
1 child	24	40,0
Order of Children in the Family		
3rd child	15	25,0
2nd child	21	35,0
1st child	24	40,0
Mother's Education		
No school, finish elementary school	8	13,3
Graduated from junior school, high school	38	63,0
Graduated Diploma / S1 / S2	14	23,3
Total Income		
Low (< US\$. 3.500/Year)	49	81,7
Hight (\geq US\$. 3.500/Year)	11	18,3
Amount	60	100

Table 2. shows the distribution of level expressions of sIgA and histatin-5 in the salivary of ECC and caries-free children. The results of statistical analysis of all ECC (31 people) and caries-free (29 people) subjects for the expression of sIgA and histatin-5 in the subjects' saliva showed a

significant correlation with caries development during examination in three repetitions ($p > 0.05$; 0.748) and the weakest correlation ($r = 0.000$). Based on the Mann Whitney-U Test analysis, the expression of sIgA and Histatin-5 on ECC and caries-free was significant ($p < 0.05$; 0.021).

Table 2. The expressions of sIgA and Histatin-5 of ECC and caries-free subjects

Group	N	sIgA ($\mu\text{g/mL}$)	Histatin-5 ($\mu\text{g/mL}$)	P value	P value
Caries-Free	N	29	29	$p < 0.05; 0.00$	$p < 0.05; 0.021$
	Mean	1,15	0,016		
	Median	0,08	0,01		
	Minimum	0,01	0,01		
	Maximum	9,87	0,04		
	Std. Deviation	1,99	0,01		
ECC	N	31	31	$p < 0.05; 0.00$	$p < 0.05; 0.021$
	Mean	0,06	2,95		
	Median	0,05	2,20		
	Minimum	0,01	0,04		
	Maximum	0,20	24,10		
	Std. Deviation	0,04	3,24		

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The expression of sIgA and Histatin in ECC ($p < 0.05; 0.00$) and Caries-free were significant ($p < 0.05; 0.00$). Meanwhile, specifically the groups that no significant were ECC with histatin-5 expression ($p > 0.05; 2.20$) also sIgA ($p > 0.05$). The concentration of sIgA and Histatin-5 also has a significant relationship with the age of ECC and caries-free children ($P < 0.05; 0.00$) with a correlation coefficient ($r = 0.83$).

Statistically, the expression of between sIgA and Histatin 5 of boys and girls groups was not significant ($p > 0.05; 0.904$) and no correlation ($r = -0.039$). It suggested that boys and girls have the same opportunity to express the sIgA and Histatin-5 in saliva during three evaluation times. sIgA and Histatin-5 expressions differ from the month periods tested (1, 3, and 9 months) but showed no any

significant difference from each examination period (1 and 3 months; $p > 0.05; 0.116$), (1 and 9 months; $p > 0.05; 0.766$), and (3 and 9 months; $p > 0.05; 0.398$).

Figure 1 showed the distribution and frequency of subjects consist of boys 47% and girls 53% with the average ages 1-12 months, 21% and 13-24 months 79%. The sIgA expression decreased at month three and increased again in month 9. In months 1 and 3, groups of boys were more dominant in expressing sIgA while at month 9, groups of boys were higher, although their expression was not significantly different. Histatin-5 expression increases at one month and decreases starting at months 3 and 9. At month one, a higher group of girls express Histatin-5, but at month three and month 9 show the same frequency

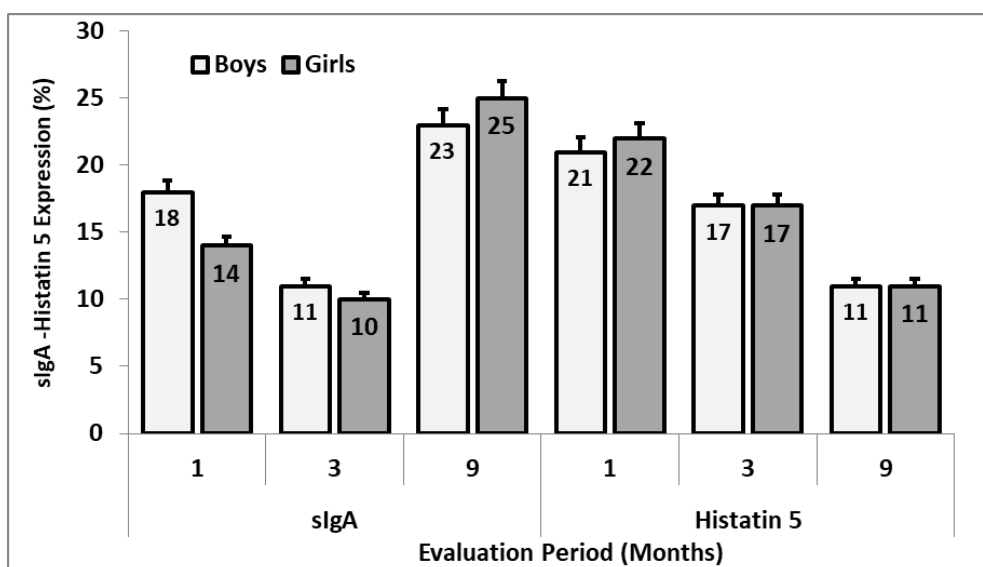


Figure 1. The profile of sIgA and Histatin-5 expressions in months (1, 3, and 9 months) from boys and girls groups. Bars (sIgA and Histatin-5 expressions) and Error bars (Error bars with percentage).

DISCUSSION

Early Childhood Caries (ECC) is a term used to describe dental caries in children aged six years or younger. Immunoglobulins and salivary proteins are known as sensitive indicators for ECC.³ Saliva, as the body's defense fluid contains various proteins, peptides, and immunoglobulins that play an essential role in preventing caries.² Identification of biomarkers for high-risk children ECC is vital for early diagnosis, but also It is crucial to prevent and treat disease.⁹ This study aimed to analyze the levels of sIgA and Histatin 5 in ECC and caries-free children.

Figure 1 shows that based on sex, the expression of sIgA and histatin 5 did not show a

significant difference. These results were in line with the research of Jaarzadeh,¹⁰ where the expression of sIgA and histatin 5 tends to have the same opportunity between men and women. Besides, the concentration of histatin-5 at the first examination (month 1) was higher than sIgA because in this condition the subject has not shown any caries intensity or any significant pathogenic. Histatin 5 is the salivary peptide that is more dominant in this phase because it is a non-antibody (innate) of the antimicrobial component.² Month-3 observations still show the same conditions as observations at month-1, where the expression of histatin-5 still dominates, but sIgA shows a decrease compared to month-1. At month-9, the expression of sIgA was higher than that of histatin-5. Similar to other

salivary antimicrobial components, the change in time is a factor that can affect fluctuations in histatin-5 and sIgA concentrations.¹¹

In addition to time and environmental factors, pathological oral factors can also cause sIgA and histatin expression.¹² As is well known, sIgA concentration is an age-dependent change, which means that salivary sIgA is usually very low in newborns, but increases rapidly. During the first month of life.¹³ Furthermore, histatin expression is induced by oral infections such as oral candidiasis and xerostomia. Environmental factors and socio-economic factors influence cases of dental caries disease in children. Table 1 shows the characteristic frequency distribution data and social data of the research subjects. In general, this data correlates with the research results shown in **Figure 1** and **Table 2**.

Table 2 shows the expression of sIgA and Histatin-5 from ECC and caries-free subjects. Caries-free children had a higher sIgA concentration compared to ECC children.¹⁴ This suggests a protective function of sIgA in preventing caries, which is in line with other studies.¹⁵ This finding is debatable because other studies have reported that caries as an infectious disease can induce an immune response to increase sIgA concentrations due to an increase in pathogens and antibody production.¹⁶ In contrast, Histatin-5 expression was higher in children with ECC. Histatin-5 and lactoperoxidase expression correlated with caries development. They are of bacteriostatic and bacteriocidal properties.¹⁷

Other studies have also stated that none of the in-vivo antibacterial components is significant in determining caries risk.¹⁸ However, these results suggest an antibacterial part of in-vivo saliva, which is substantial in explaining caries risk, namely Histatin-5. These results also indicate that the interaction between salivary protein and oral

bacteria can trigger various cellular mechanisms both in the host (saliva) and in oral organisms, one of which is the condition of host caries, increasing histatin-5 levels.^{3,19}

Early Childhood Caries shows a high number of *S. mutans* in the oral cavity. The immune response in children will respond to the high *S. mutans* antigen by producing immunoglobulins. Besides, sIgA is the front guard against this antigen.⁹ However, when the capacity of microorganisms exceeds the high level, the role of sIgA becomes inactive in fighting disease. It is related to a child. It is proven in this study that age has a significant relationship with caries or caries-free. Jafarzadeh²⁰ mentions a significant difference between sIgA in children aged 1-10 years and older subjects.¹⁰ Other research reports have shown higher sIgA levels in older subjects as well. Some results suggest this is due to the relationship between age and the cytokines produced. Nonetheless, other studies have shown that age differences do not have a significant relationship with cytokine production.

The sharing of roles between sIgA and Histatin-5 also exists in the working relationship between the two. Although the working relationship of Histatin-5 is still unclear, several studies have confirmed the action of Histatin-5 in influencing mitochondrial activity. The mechanism of Histatin-5 is through inhibiting respiration from causing cell death and loss of integrity.²² Besides, in treating caries disease, sIgA can negate several activities such as bacterial adhesion, enzyme inactivation, and bacterial toxins. Another ability is through synergistic efforts with other factors such as lysozyme, lactoferrin, histatin, mucin, and lactoperoxidase.⁹ This study indicates sIgA works synergistically with Histatin-5. The mean value of the salivary component also showed significant results in the caries-free and caries-free conditions.

CONCLUSION

In the ECC cases that occurred to decrease sIgA levels and increased of Histatin-5 also, both antibodies have expressed in Caries-free Children.

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Both sIgA and Histatin-5 involved in the pathogenesis caries infection of children.

S.D., S.H.; Materials – S.D., S.H.; Data Collection and Processing – S.D., S.H.; Analysis and Interpretation – P.A., S.Y.; Literature Search – S.D., S.H.; Writing – P.A., S.Y.; Critical Reviews – S.D., S.H.

Conflict of interest statement: The authors declare no conflicts of interest.

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