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Assessment of Salivary Immunoglobulin a and Lysozyme Levels and Their Relation to Dental Caries Status in a Group of Yemeni Asthmatic Children

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Abstract

Aim of the study: To study salivary immunoglobulin A and lysozyme levels and their relation to dental caries status in a group of Yemeni asthmatic children. ***Subjects and methods:*** One hundred children of both genders were enrolled in this study, aged 6-10 years. The cases were divided into two main groups: fifty asthmatic children and fifty healthy children. Every child was examined for dental caries and assessed according to WHO criteria (1997) using defs for primary teeth and DMFS for permanent teeth. Salivary samples were collected from each child in each group and analyzed for sIgA and lysozyme. The relation between them and dental caries status for primary and permanent teeth was evaluated. ***Results:*** Results showed that there was no statistically significant difference between gender distributions of asthmatic and healthy children. In addition, there was no statistically significant difference between the mean sIgA levels of both groups. There was a statistically significant difference between asthmatic and healthy children regarding lysozyme levels. This difference in mean lysozyme levels was higher in asthmatic than healthy children. Asthmatic children showed statistically significant differences regarding defs and DMFS scores, which were lower in asthmatics than healthy children. For asthmatic children, there was a statistically significant positive correlation between defs scores and sIgA levels and a statistically significant negative correlation with lysozyme. In asthmatic children, a statistically significant positive correlation existed between DMFS scores and age and sIgA levels. For healthy children, there was a statistically significant positive correlation between DMFS scores and both age and sIgA levels. ***Conclusion:*** In children with allergic asthma the salivary concentrations of sIgA and lysozyme were significantly higher and the defs and DMFS scores were lower than in healthy children.

Keywords: Saliva, pH, sIgA, Dental caries, Calcium, Lysozyme.

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INTRODUCTION

The risk of several oral health problems, including dental caries, can be detected as a side effect in children with chronic medical disorders that require long-term medications, such as asthma [1]. A possible mechanism for affecting oral health in asthmatic patients could be the prolonged use of bronchodilators, which may affect salivary flow rate [2].

Saliva is a watery and important physiologic fluid considered the mirror of body health. It contains highly complex mixtures, such as electrolytes, enzymes, mucus, glycoproteins, and antimicrobial compounds, like Immunoglobulin A and lysozyme [3, 4].

The lower secretion rate of whole saliva means that the availability of biologically active components, like; amylase, calcium, secretory IgA, and peroxidase is decreased. The decreased output in such antibacterial components gives the chance for both bacterial colonization and plaque growth [5].

Since asthmatic children are a large and growing proportion of pediatric dental patients, factors affecting their oral health are principal to the dental profession. So, it is crucial to investigate one of these factors to find out its relation with dental caries [5, 6].

SUBJECTS AND METHODS

Two hundred children contributed to this study; their ages ranged from 6 years to 10 years with mixed dentition. The cases were selected and divided into two main groups: 50 children healthy and 50 children asthmatic.

For asthmatics, all selected children showed Normal patterns of growth and development, absence of congenital or systemic disease except as match children, absence of dental abscess, or any mucosal lesions.

For healthy, all selected children showed Normal patterns of growth and development, absence of congenital or systemic disease, lack of any medical treatment, absence of dental abscess or any mucosal lesions.

For all children previously diagnosed as asthmatic, a questionnaire was utilized to register information about the type and duration of the medication used.

Dental Examination:

At the first dental appointment, the children's parents were informed about the purpose and procedure of the study. A written informed consent was taken before the clinical examination and investigation.

Complete demographic data was collected for each case, including the name of the child, age, gender, address, parent's education level, socioeconomic status, parent's career, family history, and medical and dental history [8].

Complete extra-oral and intra-oral examinations were carried out, including examination of hard and soft tissues, information on the time of eruption of different teeth (sequences of tooth eruption), and the number of teeth presented was counted at the time of eruption (done separately for primary and permanent teeth), evaluation of oral hygiene status. Dental caries was assessed for each child according to WHO criteria (1997) for detection of caries using DMFS (decayed, missing, and filled surfaces) for permanent dentition, and defs (decayed, decayed tooth indicated for extraction, filled surfaces) for deciduous dentition [9, 10].

At the end of the clinical examination, all children received instructions about oral health maintenance and how to brush their teeth. Children who fulfilled the criteria of the study were included while others were excluded from the study but treated or referred for treatment [10].

The dental examination for healthy children was carried out in the dental chair in the dental pediatric clinic and was performed using a sterilized dental mirror and explorer (visual and tactile method) and radiographically using bitewing radiographs.

For asthmatic children, the dental examinations were carried out at the pediatric clinic in the chest hospital using disposable dental examination instruments. In case of doubtful caries status, they were subjected to radiographic examination at the same hospital.

Salivary sample collection:

The child received detailed information about the collection protocol; to exclude brushing teeth immediately before collection, and to avoid food or fluid (apart from water) ingestion or chewing gum at least 1 hour before the sample collection. At the clinic, the child was instructed to brush his teeth with a toothbrush without paste and rinse his mouth with distilled water at least 10 minutes before the collection of the sample. Unstimulated whole saliva was collected in a wide-mouth sterilized test tube in the morning hours between 9 am to 12 pm. The collection of samples took 5-10 minutes. The collected samples were kept in an ice box submersed in dry ice and transported to the laboratory.

Laboratory procedures:

At the lab, the sample was centrifuged at 4000 rpm for 20 minutes to eliminate cellular debris. The supernatant was separated by a dropper put in epundorf and stored at -70ºc (Figure 1).

Figure 1: Samples were centrifuged at 4000 rpm for 20 minutes

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Figure 2: Collected samples stored in epundorf at -70ºc

1- Measurement of sIgA:

Secretory IgA concentration in saliva was measured by enzyme-linked immunosorbent assay (ELISA) * (Figure 3).

The kit contained: microtiter strips, six calibrators, two controls, antibody solution, enzyme conjugate, sample buffer, wash buffer10×, substrate, and stop solution.

The sIgA was measured according to the instructions of producers as follows:

The samples were diluted 1:201 sample buffer (5µl sample to 1.0ml buffer) and mixed.

The samples were incubated by transferring 100µ of the calibrators, controls, and diluted samples into the individual microplate wells, 100µ of enzyme conjugate solution was pipetted into each of the microplate wells and incubated for 60 minutes at room temperature (Figure 4). The wells were emptied and washed automatically 3 times with 450µ working strength wash buffer, 100µl of chromogen/substrate solution was pipetted into each of the microplate wells and incubated for 10 minutes at room temperature and protected from direct sunlight, 100µl of stop solution was pipetted into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced. Before measuring, the microplate was slightly shaken to ensure a homogeneous distribution of the solution, Photometric measurement of the color intensity was made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm within 30 minutes of adding the stop solution. The results were calculated by the standard curve from which the concentration of antibodies in the samples was obtained; by point-to-point plotting of extinction values measured for the 6 calibration sera against the corresponding units (linear/log). "4-parameters, logistics "plotting was used for the calculation of the standard curve by computer (Figure 5).

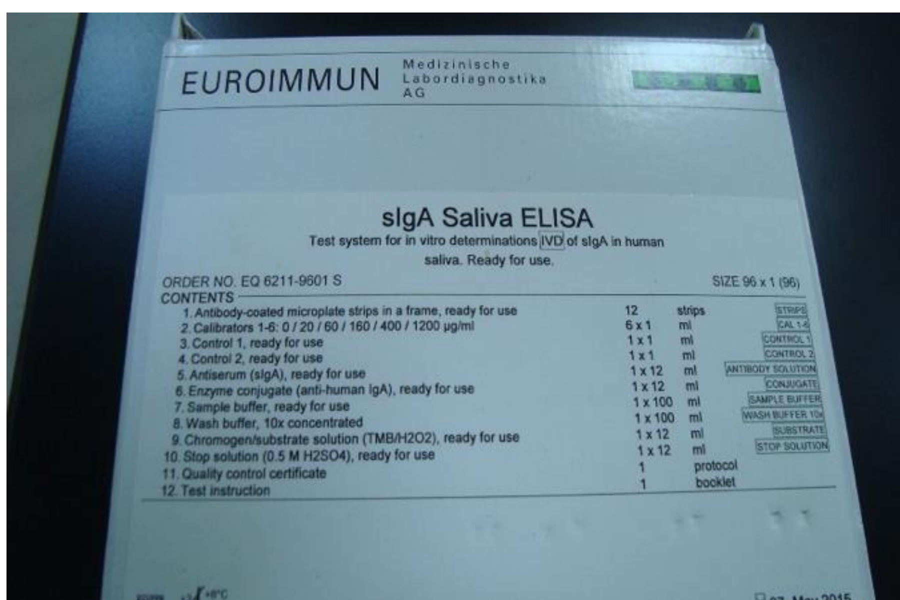

Figure 3: Secretory IgA ELISA kit

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Figure 4: Patient samples in microplate

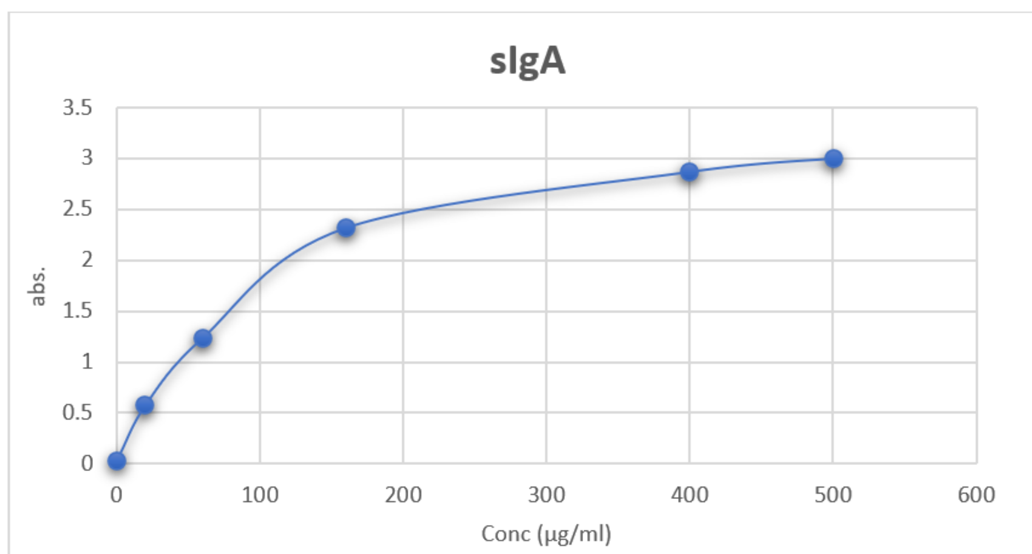

Figure 5: Standard curve for the concentrations and the corresponding absorbance of sIgA

2- Measurement of lysozyme:

Lysozyme concentration, also, was measured by ELISA [**] (Figure 6). The kit contained: microplates, standard, diluent N concentrate, biotinylated human lysozyme antibody, conjugate, chromogen substrate, stop solution, wash buffer concentrate, and sealing tapes. The lysozyme was measured according to the instructions of producers as follows:

> All materials and prepared reagents were equilibrated to room temperature before use. The samples were diluted 1:8,000 into 1×Diluent N and assayed. Excess microplate strips were removed from the plate frame and returned immediately to the foil pouch with desiccant inside. The pouch was resealed securely to minimize exposure to water vapor and stored in a vacuum desiccator. 50µl of lysozyme standard or sample was added per well and incubated for two hours. The wells were washed five times with 200µL of 1× wash buffer manually. 50µl Biotinylated lysozyme antibody was added to each well and incubated for one hour. The microplate was washed as described above and 50 µl of conjugate was added to each well and incubated for 30 minutes. 50 µl of conjugate to each well was added and incubated for 30 minutes. Again, the microplate was washed and 50 µl of chromogen substrate was added to each well and incubated for 15 minutes. 50 µl of stop solution was added to each well and incubated for 15 minutes. The absorbance was read on a microplate reader at a wavelength of 450nm. (Figure 8). The mean value of the reading for each standard and

sample was calculated. To generate a standard curve, the curve was plotted using the standard concentrations and the corresponding absorbance (Figure 9).

Figure 6: Lysozyme human ELIZA kit

Figure 7: Microplate reader

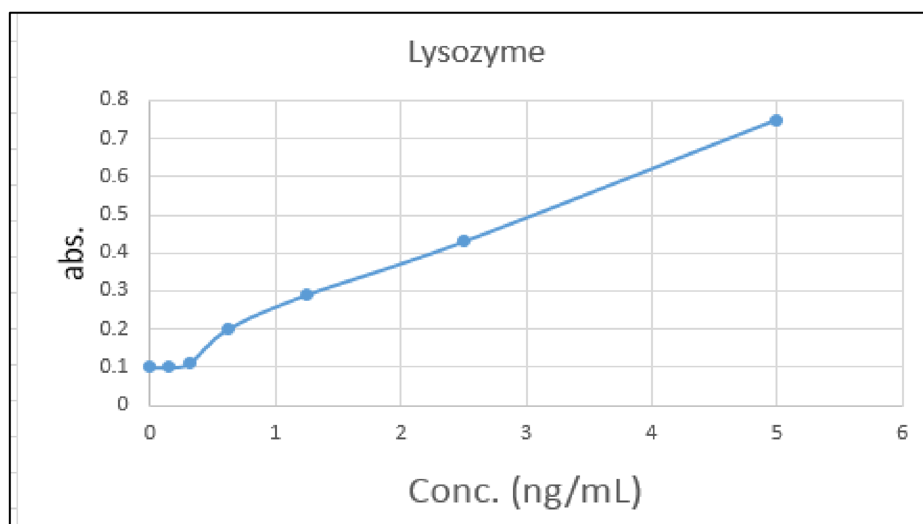

Figure 8: Standard curve for the concentrations and the corresponding absorbance of lysozyme

RESULTS

1. Comparison between Asthmatic and Healthy Children

1.1. Gender

There was no statistically significant difference between gender distributions of asthmatic and healthy children as shown in Table (1).

1.2. Secretory Immunoglobulin A (sIgA)

Table (2) clarifies that there was no statistically significant difference between mean sIgA levels of asthmatic and healthy children.

1.3. Lysozyme:

In addition, Table (2) shows that there was a statistically significant difference between healthy and asthmatic children with *p-value = 0.054*. This difference in mean lysozyme levels was higher in asthmatic than healthy children.

Table 1: Gender distributions between Asthmatic and Healthy Children

Gender	Asthmatic		Healthy		*P*-value
	N	%	n	%	
Boy	28	56.0	28	56.0	1.000
Girl	22	44.0	22	44.0	

: Significant at $P \leq 0.05$

Table 2: Comparison between sIgA and lysozyme levels in asthmatic and healthy children

Variable	Asthmatic		Healthy		*P*-value
	Mean	SD	Mean	SD	
sIgA	308.00	111.01	257.80	113.66	0.073
lysozyme	5.41	2.45	4.79	1.98	0.054*

: Significant at $P \leq 0.05$

1.4. DEFS Scores

Children showed statistically significant differences with ***p values = 0.021*** between healthy and asthmatics**.** The mean defs scores were lower in asthmatics than in healthy, as shown in Table (3).

1.5. DMFS scores

Table (3) compares healthy and asthmatic children and clarifies that asthmatic children showed statistically significantly lower mean DMFS scores than healthy children with *p-value =0.006*.

Table 3: Comparison between defs and DMFS scores in asthmatic and healthy children

Variable	Asthmatic		Healthy		*P*-value
	Mean	SD	Mean	SD	
defs	6.00	5.01	8.88	6.47	0.021*
DMFS	0.84	1.23	1.96	2.17	0.006*

: Significant at $P \leq 0.05$

2. Correlation between defs scores and different variables:

2.1. Asthmatic group

Results demonstrated that there was a statistically significant positive correlation between defs scores and age. In addition, there was a statistically significant positive correlation between defs scores and sIgA levels and a statistically significant negative correlation with lysozyme as shown in Table (4).

2.2. Healthy group

On the other hand, table (4) shows that there was a statistically significant negative correlation between defs scores and age. There was no statistically significant correlation between defs scores and other variables (sIgA and Lysozyme).

Table 4: Correlation between defs scores and different variables

Variables	Asthmatic		Healthy	
	Correlation coefficient	*P*-value	Correlation coefficient	*P*-value
defs and age	0.291	0.040*	-0.405	0.004*
defs and sIgA	0.497	<0.001*	-0.176	0.221
defs and lysozyme	-0.412	0.003*	-0.236	0.099

: Significant at $P \leq 0.05$

3. Correlation between DMFS scores and different variables

3.1. Asthmatic group

Table (5) clarifies that there was a statistically significant positive correlation between DMFS scores and age. There was a statistically significant positive correlation between DMFS scores and sIgA levels. On the other hand, there was no statistically significant correlation between DMFS scores and lysozyme.

3.2. Healthy group

Table (5) also, shows that there was only a statistically significant positive correlation between DMFS scores and both age and sIgA.

Table 5: Correlation between DMFS scores and different variables

Variables	Asthmatic		Healthy	
	Correlation coefficient	*P*-value	Correlation coefficient	*P*-value
DMFS and age	0.585	<0.001*	0.678	<0.001*
DMFS and sIgA	0.286	0.044*	0.283	0.047*
DMFS and lysozyme	0.108	0.455	-0.219	0.127

: Significant at P ≤ 0.05

DISCUSSION

The use of saliva has provided a considerable addition to the diagnostic armamentarium as an investigative tool. It provides important information about the functioning of various organs within the body and is mainly utilized for research and diagnosis purposes concerning systemic diseases.

This study aimed to study the variations in some salivary parameters (sIgA and lysozyme) and their influence on dental caries status in asthmatic and healthy children.

Saliva has the advantages of being simple, noninvasive in collecting method, safe for the operator and the patient, and easy and low-cost storage. These positive characteristics of saliva make it possible to monitor several biomarkers in infants, children, elderly and non-collaborative subjects [11].

In this study, asthmatic children between the ages of 6 and 10 years were chosen, as asthma is one of the most common chronic diseases among Children and reaches its peak of prevalence at this age [12,13]. In addition, the selected children were free from any other systemic congenital diseases and lack of any medication therapy (only for healthy). These criteria were based on the fact that saliva biochemical composition is affected by chronic diseases, infections, and medications [14].

In this study, there was standardization in saliva collection to prevent several factors affecting salivary flux and composition. The time of saliva collection was in the morning hours between 9 am to 12 pm to prevent any differences in the concentration of saliva due to the circadian rhythm.

Whole saliva was collected, which is easy to collect and more representative of the oral environment, and the collection does not need to be performed by a trained specialist but can be made by the study participant [11]. Additionally, unstimulated saliva was collected because the studied parameters (sIgA and lysozyme) are found in greater quantities in unstimulated than in stimulated saliva, as their main source are the submandibular and sublingual glands [15, 11].

The saliva samples were kept in an icebox as soon as possible after collection to maintain the sample integrity. The collected salivary sample was centrifuged at 4000 rpm for 20 min, to avoid visible precipitates. It was then stored at -70°c to prevent the degradation of some molecules in saliva and prevent bacterial growth [11, 16, 7, 17].

First of all, the result showed that the distribution of age categories was the same in both groups this reflects the proper selection of the study groups.

1. Comparison between asthmatic and healthy children in each group

1.1. Secretory IgA (sIgA):

According to the results of this study, asthmatic children showed a higher sIgA level than healthy children. This finding was truly supported by *Rashkova et al. 2009* who quantified sIgA in the saliva of children with type 1 diabetes, asthma, and no systemic disease but wearing a removable orthodontic appliance. The results indicated that children with asthma showed statistically significant higher values of sIgA [18].

The higher level of sIgA in asthmatic than healthy children may be due to asthma as the disease characterized by chronic airway inflammation and congestion. The initial stimulation of secretory immunoglobulin-expressing B-cells takes place in mucosa-associated lymphoid tissues, mainly nasopharynx-associated lymphoid tissues (NALT), which orchestrate regional immune functions against both airborne and alimentary antigens. This regional immune function is destined for the salivary glands and oral mucosa, resulting in local reaction and secretion of sigA [7, 18-20].

1.2 Lysozyme:

According to the result of this study, asthmatic children showed statistically significantly higher lysozyme levels than healthy children. This result was in agreement with *Hyyppa et al., 1981* who assessed IgE, lysozyme, histamine concentration, and aminopeptidases in asthmatic and control groups. They concluded that both histamine and lysozyme were increased in the asthmatic than in the control group. Although the study group was adult. They demonstrated that the high level of lysozyme might be due to the fact that as a result of a local allergic reaction, the mucosal epithelial cells, sub-mucosal tissue, and macrophages all secret lysozyme and the elevated salivary lysozyme concentration may be in part due local enrichment or secretion of the protein. In addition, lysozyme was suggested as a natural antihistamine [21, 22].

1.3 defs:

Our finding demonstrated statistically significant lower defs score in asthmatic children than healthy children. This result was different from those of *Shulman 2001*, who found no statistically significant difference between asthmatic and control children regarding dfs score. This may be due to the different indices used in the two studies [23].

Also, this study was not in agreement with *McDerra et al., 1998* who demonstrated a significant difference between asthmatic and healthy children but with higher levels of caries experience in asthmatic than healthy children. The differences between the two studies may be related to different socio-economic statuses, as the *MccDerra* study was carried out in Britain. This study was carried out in developed countries with low socioeconomic populations and a lack of healthcare resources, so they are not able to afford asthma medications regularly, and in turn, the effect of asthma medication was not obvious [24].

1.4 DMFS:

The results showed a statistically significant lower level of DMFS score in asthmatic than healthy children. This result was in agreement with *Shulman et al 2001* who studied the association between childhood asthma and caries; they compared the DMFS score between asthma and the control group. They found that children with asthma had significantly lower DMFS scores and this result may be related to the severity of the disease [23].

CONCLUSION

1. Salivary analysis revealed an overall altered salivary composition in children with asthma, indicating a compromised oral environment in these patients and suggesting salivary analysis as an additional diagnostic tool for allergic diseases.
2. In children with allergic asthma the salivary concentrations of sIgA and lysozyme were significantly higher and the DMFS scores were lower than healthy children.
3. Study of different populations which exhibit distinct caries prevalence rates, could provide a useful descriptive measure of caries susceptibility in tooth surfaces.

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