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The differential electrophoretic patterns of statherin and histatins in caries-active and caries-free children

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Abstract
Aim: The present study aimed to reveal the possible different protein profiles of children who had caries-active and caries-free deciduous dentition. Salivary parameters including salivary pH, flow rate, the concentrations of calsium, phosphate and total protein, were also evaluated to see the possible changes in case of dental caries.
Materials and methods: Eight subjects were participated in 'caries-active group' and the other 10 subjects were in 'caries-free group'. Salivary parameters including salivary pH, flow rate and the concentrations of calcium, phosphate and total protein were evaluated in both groups and the electrophoretic patterns for Statherin and Histatins in the groups were analysed by using Discontinuous Native Polyacrylamide Gel Electrophoresis and Basic Gel Electrophoresis; respectively.
Results: The slight differences were observed on the salivary parameters of the groups (p>0.05). Salivary proteins were identified according to their relative mobility in gel and stain patterns. A total of 7 Statherin (38.8%) and 3 Histatin1 (16.6%) bands were counted on the gels. All 3 Histatin1 bands were seen in only caries-free samples meanwhile 6 of 7 Statherin bands (33.2%) were in caries-free samples, as well.
Conclusion: These preliminary findings report that satatherin and histatin1 had different electrophoretic patterns in children with different caries status. Additional studies can provide further evidence concerning the role of each salivary proteins in modifying risk for dental caries.

Keywords: Children**,** dental caries, gel electrophoresis, histatins, statherin, saliva

1. Introduction
Dental caries is a chronic disease characterized by demineralization of tooth structure. The process initiated within only biofilm-covered tooth surfaces by acidic end-products of dietary carbohydrates. The management of dental caries is dependent on the assessment of caries risk because it is preventable and initially reversible [1]. Host factors play an important role in caries risk assessment including salivary parameters such as composition and concentration of its organic/inorganic elements and also flow rate of this fluid [2]. As the flow rate changes; pH and the concentrations of organic/inorganic elements, including calcium, phosphate and total protein, change as well [3]. The chemical composition of saliva is believed to be associated the presence of tooth decay. Related to this field, there have been numerous attempts to show an association between dental caries and the amount of calcium in saliva [4, 5]. Fewer studies have considered the phosphorus content as well [6, 7].
Saliva is also thought to be a natural protective mechanism against tooth decay [8] because it contains salivary proteins which adsorb strongly onto the teeth, protecting enamel against acid dissolution. This adsorbed protective layer is called acquired enamel pellicle (AEP) and acts as a selective permeable barrier that regulates mineralization/demineralization processes. It also controls the composition of the microbial flora that form dental plaque [9]. Recent studies using human whole saliva have shown individual differences in salivary protein patterns [8, 10]. Genetically determined variations in salivary protein composition may play an important role in the etiology of dental caries and other oral diseases [11]. It has thus been agreed that determining salivary protein profiles of children can contribute to the evaluation of caries risk and hence early prevention of this widespread disease [8, 10]. The *in vivo* AEP showed significant numbers of histatins (histatin1, 3, and 5) remain intact [12]. When histatins are adsorbed onto the enamel surface forming the AEP, these proteins provide protection against acid injury [12]. Histatin 1 is a salivary protein that also exhibits antifungal and antibacterial

activities and has functions include buffering and modulation of mineral formation [13]. Similar to Histatins, Statherin is a small molecular weight salivary protein with negative net charges that inhibits both primary and secondary calcium phosphate precipitation [14].

Native Polyacrylamide Gel Electrophoresis (Native PAGE) and Basic gel electrophoresis are the techniques for separating biologically active proteins. In contrast to SDS-PAGE (Sodium dodecyl sulphate- Polyacrylamide Gel Electrophoresis), the mobilities of proteins in a native PAGE and Basic gel systems depend on both size and charge. In electrophoresis, proteins are separated on the basis of charge, and if the electrodes are arranged in such a way that the upper bath is - (cathode), while the lower bath is + (anode), and - anions are allowed to flow toward the anode, the system is known as an anionic system (Discontinuous Native PAGE). Flow in the opposite direction is a cationic system (Basic Gel Electrophoresis) and allows + cations to move toward the cathode.

In the present study we aimed to reveal the possible different electrophoretic patterns of salivary proteins; Statherin and Histatins in children having caries-active and caries-free deciduous dentition by using Discontinuous Native-PAGE and Basic Gel Electrophoresis. In addition salivary parameters including salivary pH, flow rate and the concentrations of calcium, phosphate and total protein were evaluated in both groups, as well.

2. Materials and Methods

2.1. Subject selection

The study protocol was approved by the Research Human Ethics Board of Western University (Review number; 16181E). The caries status of children aged 3-5 years old were assessed according to the International Caries Detection and Assessment System (ICDAS II) criteria. 'Caries-active group' was composed of 8 children having at least two active caries lesions and 'caries-free group' was composed of 10 children having no caries and/or fillings. Children who were participated in the study, were free from systemic or local diseases which affect salivary secretions. Children who have been on medication for the last 3 months that could affect the saliva composition and children who had topical fluoride application in the last 6 months were not included.

2.2. Saliva collection

Children were asked to refrain from any consumption of food, beverages and dental hygiene products for at least 1 hour prior to collection time which is necessary for saliva to return to the resting state. Unstimulated whole saliva samples were collected in ice at 9:00-11:00 am to avoid the circadian rhythm effects on saliva composition and flow rate. All procedure was done with the patient comfortably seated in a well-ventilated and lighted room. To standardize the saliva collection from the children aged 3-5 years we used an eppendorf tube and a suction device which creates a negative pressure in the tube, instead of using 'spitting' and/or 'drooling' methods.

2.3. Salivary Analysis

The time and volume of collected saliva were recorded to measure the salivary flow rate. Then the collected saliva samples were divided into 2 halves; the half was kept as whole saliva and used for measurement of salivary pH, Ca and phosphate concentrations, meanwhile the other half was centrifuged at 14,000× *g* for 20 min and the supernatants were separated from the pellets to use for the determination of total protein concentration. First salivary pH was measured by pH metre (SympHony SB70P, VWR, US) with micro pH electrode (PHR-146, Lazar, CA, USA). Quantitative colometric calcium determination was carried out with QuantiChrom-TM Calcium Assay Kit (DICA-500, BioAssay Systems, Hayward, CA, USA) which was used according to manufacturer's instructions. Phosphate concentration was determined by performing a colometric method (Gee-Deitz) which allows to quantify the color reaction between working reagent and known sample/diluted standard solution (KH_2PO_4) at the wavelenght 415nm [15]. Bicinchoninic acid (BCA) protein assay kit with bovine serum albumin standard (Pierce Chemical, Rockford, IL, USA) was carried out to measure the total protein concentration of each subject at 562nm (BioRad iMark Microplate Absorbance Reader, CA).

30 µg/µl of whole saliva supernatant for each sample was subjected to Discontinuous Native PAGE by using Statherin (4 µg/µl) as standards, and Basic Gel Electrophoresis by using Histatins (3 µg/µl of Histatin 1, Histatin 3 and Histatin 5) as standards. The concentrations of ingredients for separating and stacking gel solutions were given in Table 1.

Table 1: The used concentrations of separating and stacking gel solutions for Discontinuous Native-PAGE and Basic Gel Electrophoresis

Separating gel solutions;		**Stacking gel solutions;**	
For 10 mL 12% Discontinuous Native PAGE		**For 10 mL Discontinuous Native PAGE**	
Distilled deionized water	3.500 mL	Distilled deionized water	3.500 mL
Acrylamide/BIS (30%)	4.000 mL	Acrylamide/BIS (30%)	4.000 mL
Tris-HCl 1.5 M pH 8.8	2.500 mL	Tris-HCl 1.5 M pH 8.8	2.500 mL
APS 10% (w/v)	0.050 mL	APS 10% (w/v)	0.050 mL
TEMED	0.005 mL	TEMED	0.010 mL
For Basic Gel Electrophoresis		**For Basic Gel Electrophoresis**	
Acrilamide/BIS (30%)	2.000 mL	Acrilamide/BIS (30%)	0.500 mL
0.5 g Amonyum persulphate, 9 mg Riboflavin 5'phosphate Final volume 5 mL	0.015 mL	24 mL 1N KOH, 22.5 mL distilled water, 0.5 mL TEMED. pH 5.9 (adjusted with glacial asetic acid)	0.500 mL
12 mL 1M KOH, 12.5 mL distilled water, 120 µL TEMED, 25 mL glacial asetic acid. pH 2.74 (adjusted with 45% KOH)	2.000 mL	10 g sucroz Final volume 25 mL Distilled water 4 mg Riboflavin 5'phosphate (final volume 50 mL)	2.000 mL 0.500 mL 0.500 mL

2.4. Statistical analysis

The gels were scanned (FX- Bio-Rad, Hercules, CA, USA), and statistical analysis was performed using a commercially available software program IBM SPSS Statistics 22 (IBM SPSS, Turkey). Student's t test was used to compare the groups. Statistical significance was accepted for $p < 0.05$.

3. Results and Discussion

Eighteen children (10 girls, 8 boys) aged 3-5 years old (mean ± SD; 46.8 ± 9.5 months) participated in this study. We collected unstimulated whole saliva to eliminate the differences in the compositions of each gland's secretions when they are stimulated [16].

The slight differences were observed on the concentrations of calcium (5.98 ± 1.38 mg/dL in caries-free group; 6.01 ± 1.32 mg/dL in caries-active group) and phosphate (4.33 ± 1.24 mM in caries-free group; 5.92 ± 2.26 mM in caries-active group) between the groups, but these differences were not statistically significant (Table 2). In addition to saliva's protective role mediated by its ability to clear cariogenic substances from the mouth, saliva is also the primary resource of calcium and phosphate which are necessary to remineralize the enamel. But, conflicting results have been obtained from the studies investigating the calcium and phosphate contents of saliva and their relations to dental caries [7, 17, 18]. The present study showed that the salivary calcium concentration in caries active group was higher than which was found in caries free group, but the difference was not significant. Horton *et al*. analyzed saliva samples of several hundred children with varying numbers of carious teeth, and then reported that as the number of carious teeth increases, the calcium concentration of the saliva decreases [19]. They also observed higher salivary calcium concentrations at the early stages of caries development. Because in our study, caries-active group were mostly composed of white spots lesions which are the initial step of dental caries, thus lead us to suggest that the higher calcium levels found in our caries active group might be due to the high number of white spot lesions presented in the children with caries-active dentition.

In the studies with phosphorus levels of the saliva, Hawkins found lower levels in caries free individuals than for those who were susceptible to caries, while Karshan *et al*. found it higher in caries-free group [20, 21]. In the present study we did not found any statistical differences between the groups. The limited study population of the present study was the most likely the reason for the lack of differences.

The saliva flow rates and salivary pHs in both groups were shown in Table 2 with statistical p values. The outcome of the present study showed that unstimulated whole saliva pH had a weak correlation with caries activity. An increase in the salivary flow rate was observed among both the groups with a decreased protein concentration indicating an inverse relationship between the salivary flow rate and protein concentration due to the protein dilution levels in saliva (Figure 1). However higher total protein concentration was found with lower salivary pHs (Figure 2). Similar results with the present study were seen in the studies conducted by Birkhed [22] and Heintze [23] showing that no correlation was found between salivary flow rate and caries activity. However in the studies of Browne *et al*. [24] and Scully [25], it was shown that dental caries is probably the most common consequence of hypo-salivation. Because the normal range of saliva flow can be very large and may include individuals with very slow flow rates who do not have any problems like dry mouth [26], it can be suggested that the measurement of salivary flow rate would provide valuble meanings when it was measured at appropriate intervals.

Table 2: Salivary parameters in caries free and caries active children

	Flow rate (mL/min)	Salivary pH	Phosphate (mM)	Total protein (µg/mL)	Calcium (mg/dL)
Caries-Free	0.359±0.20	7.15±0.15	4.33±1.24	647.85±159.92	5.98±1.38
Caries-Active	0.455±0.24	7.03±0.38	5.92±2.26	782.10±240.02	6.01±1.32
p	0.207	0.389	0.389	0.389	0.324

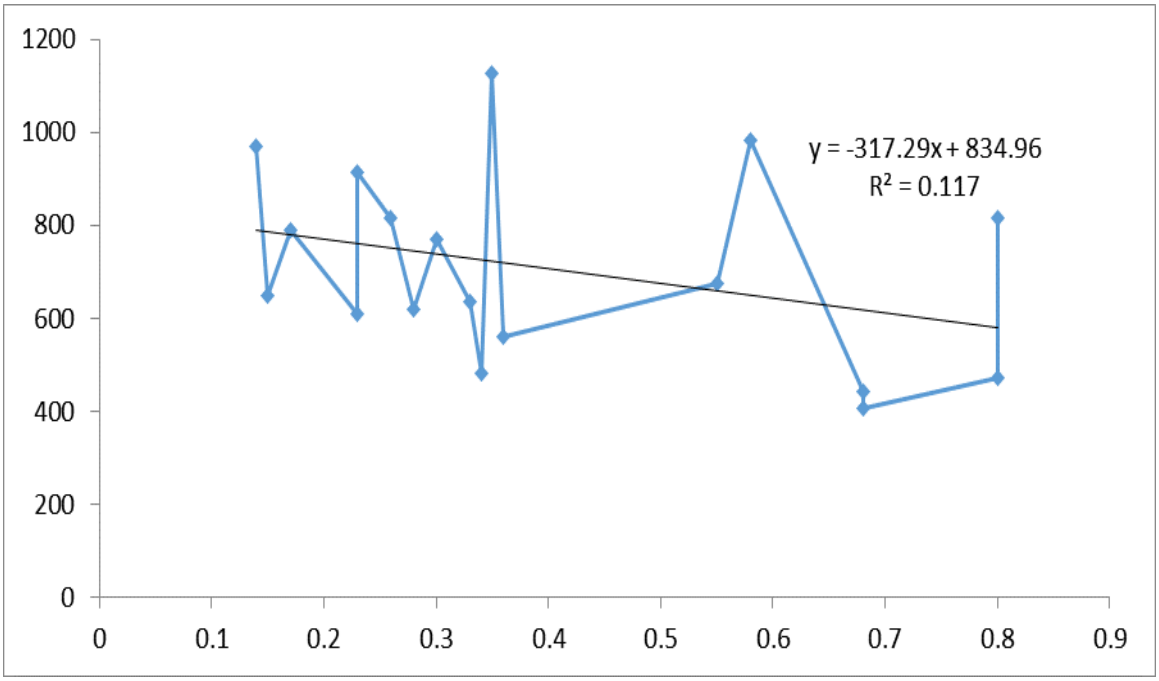

Fig 1: The association between total protein concentration (µg/mL) and saliva flow rate (mL/min) of 18 samples

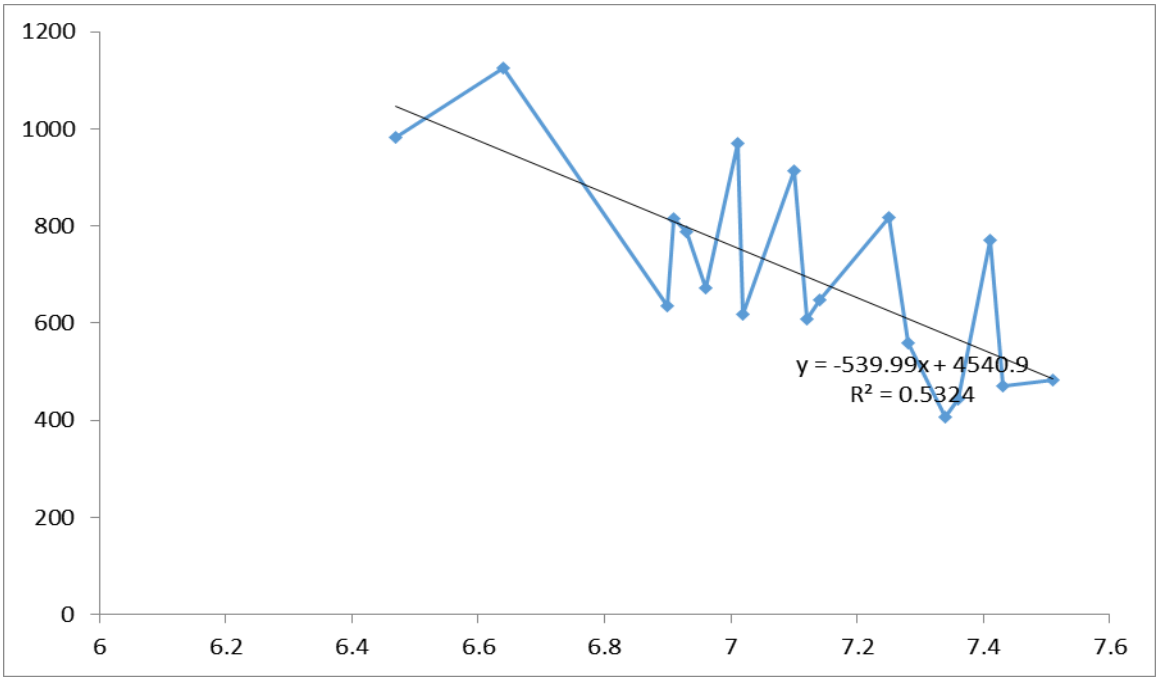

Fig 2: The association between total protein concentration (µg/mL) and salivary pH

The total protein concentrations of the groups presented different protein profiles (647.85 ± 159.92 µg/mL in caries-free group; 782.10 ± 240.02 µg/mL in caries-active group). These results are similar to Vitorino *et al*., who found a higher number of total protein concentration in caries active group [27]. It was suggested that increased proteolytic activity or decreased anti-proteolytic processes of caries-active individuals could be associated with higher protein concentration found in caries-active group. Therefore the higher protein concentration with a lower pH level in saliva of caries-active children would be a protective response of the body. We also observed an inverse association between the salivary flow rate and protein concentration. These results were consistent with the reports of Cohen *et al*. [28] and Bhalla *et al.* [29] who had explained an increase in salivary flow rates resulting in decreased protein concentrations due to protein dilution in saliva.

Dental caries is one the most common chronic diseases throughout the lifetime [30]. This condition is regulated by multifactorial factors as example, salivary proteins. During the past years important studies have been made in the separation and characterization of the salivary proteins by using the technique of electrophoresis on polyacrylamide gel. Saliva is a complex oral fluid contains a wide spectrum of proteins, namely, amylase, mucins, histatins, statherin, proline-rich proteins and others [9]. Some of these proteins have shown to be affected in maintaining the health of oral cavity and there have been many studies attempting to relate dental caries and salivary proteins [31, 32]. Therefore in the current study we analysed saliva samples with different gel matrix buffers and electric currents by using Discontinous Native-PAGE and Basic Gel Electrophoresis to see the electrophoretic patterns of differently charged proteins; Statherin and Histatins. According to our results, Statherin and Histatin1 bands were seen in caries free samples with a high trend. Salivary proteins were identified according to their relative mobility in gel and stain patterns. The number of bands presented for each subject was counted on the gels according to the band size and stain intensity as absent, present, and high intensity and size (Figure 3 and 4). In the Discontinuous Native PAGE and Basic Gel Electrophoresis salivary proteins were scored only as present and absent. According to the presence or absence of stains, a total of 7 Statherin bands (38.8%, analysed by Discontinuous Native PAGE) and 3 Histatin1 bands (16.6%, analysed by Basic Gel Electrophoresis) were counted in all samples. But Statherin bands were scored as 60% in caries-free samples whereas 12.5% in caries-active samples. In addition, Histatin1 bands were scored as 30% in only caries-free samples (Table 3). Statherin and histatins are known as the precursor proteins of acquired enamel pellicle which regulates mineralization/demineralization processes. Statherin has various functions as binding with high selectivity to hydroxyapatite and promote crystal inhibition of supersaturated calcium and phosphate in saliva, that in turn facilitates enamel remineralization [33]. It also has functions on the colonisation of initial microbial layer on tooth surface [14]. Similar to statherin, histatins are salivary proteins that are adsorbed onto the enamel surface to form the AEP [12]. In addition, histatins when adsorbed onto the enamel surface provide protection against acid injury. Our results support the studies emphasizing those Statherin is determinant of initial microbial colonization of tooth surface and histatins have functions as antifungal and antibacterial *in vivo* [34, 35].

Table 3: The distribution of bands counted in gels according to groups

	Caries-free samples (10 subjects)	**Caries-active samples (8 subjects)**	**Total 18 subjects**
Statherin	6 bands (60%)	1 band (12.5%)	7 bands (38.8%)
Histatin1	3 bands (30%)	-	3 bands (16.6%)

4. Conclusion

Although these findings are preliminary, we suggest that there is a kind of trend in electrophoretic patterns of Statherin and Histatin1 in case of dental caries. Further information on molecular epidemiology of salivary proteins may support the use of this methodology as a diagnostic tool in dental caries and other oral health problems. At this time, the data generated herein is extremely relevant to the foundation for the new diagnosis and prevention methods of dental caries by detection and quantification of a single biomarker.

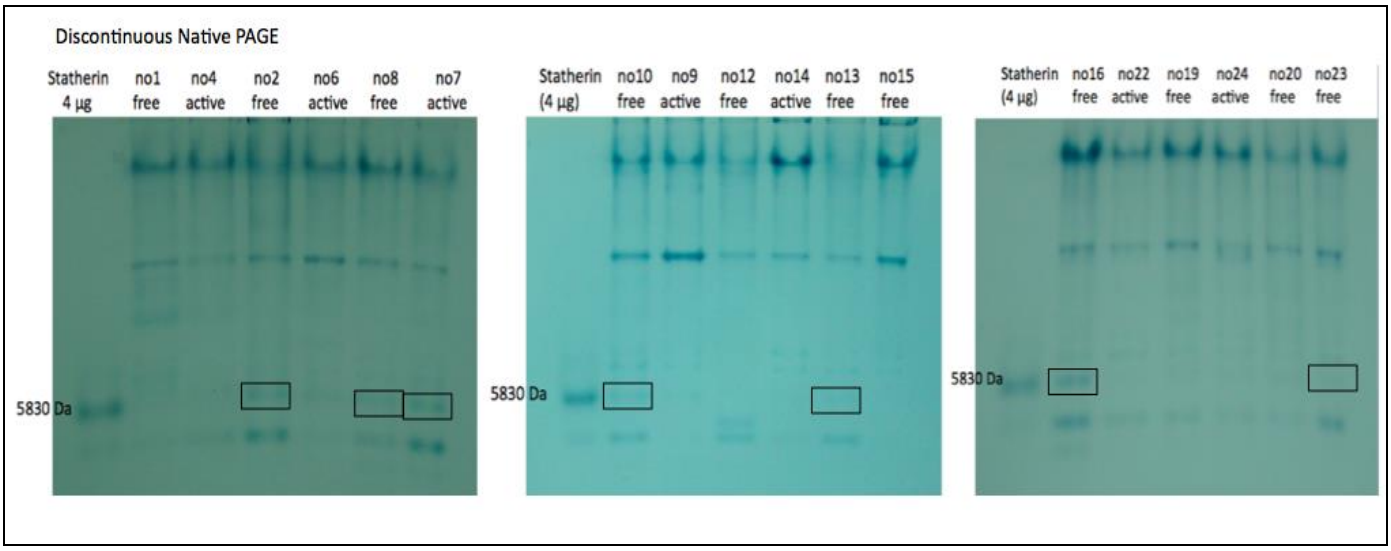

Fig 3: Gel and stain patterns of Discontinuous native – PAGE; Statherin bands presented with rectangular frames, were seen in 6 caries-free and 1 caries-active samples

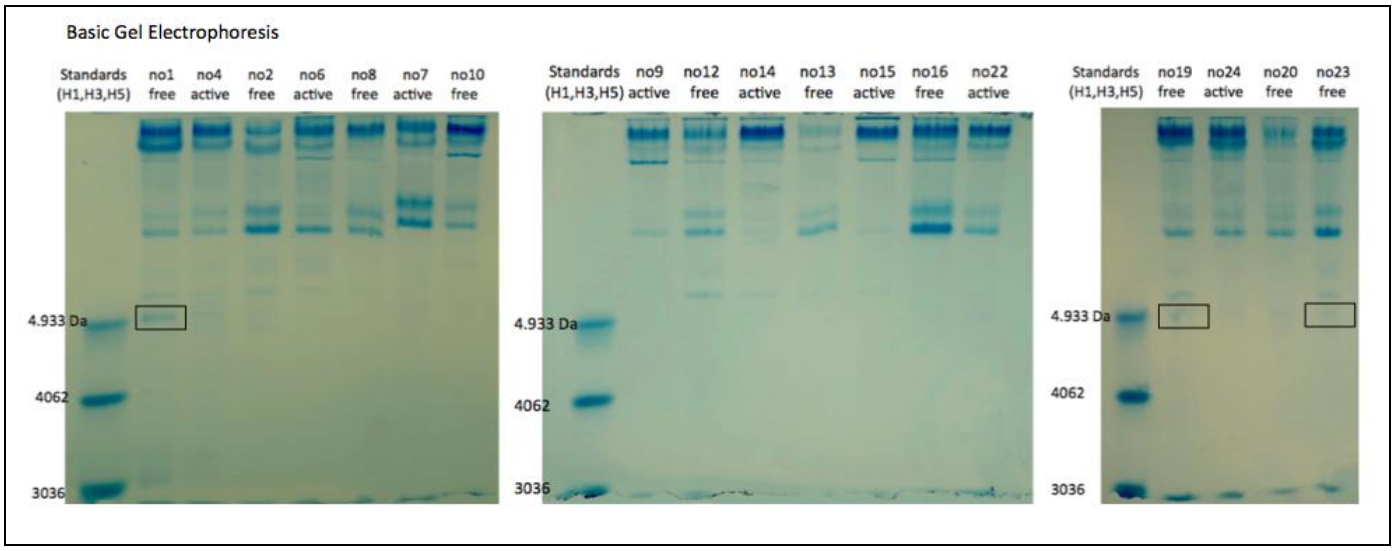

Fig 4: Gel and stain patterns of Basic Gel Electrophoresis; Histatin1 bands presented with rectangular frames, were seen in only 3 caries-free samples

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