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The Phenotypic Polymorphism of Saliva From Dental Caries Patients

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Abstract: Introduction: The tooth decay or dental caries is a disease that results in imbalance in mineralization process of the tooth, cavitation and sensitivity as well as pulp infection that leading to inflammation and eventually tooth loose. Saliva which is in direct contact with the dentine, can serve as a medium for analysis of components or factors that show the difference in normal healthy and disease conditions. **Methodology**: Here, a biochemical analysis of individual saliva samples from healthy control and caries patients were carried out to see the differences in protein profiles. The unstimulated saliva samples were analyzed through sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), two-dimensional gel electrophoresis (2D gel), zymography and secretary IgA (sIgA) antibody ELISA procedure. **Results**: Differences were observed in individual samples not only in polymorphic band pattern on SDS-PAGE gels but also in activity of proteases in the saliva and the concentration of sIgA antibody. **Conclusion**: These were the initial results obtained from the study that was further optimized and evaluated with accuracy and reported elsewhere with statistical figures.

Keywords: Saliva, Caries, SDS-PAGE, Zymography, ELISA, sIgA.

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Introduction

The common diseases and disorders of a human body are usually diagnosed by analyzing the body fluids such as blood, cerebrospinal fluid, urine, sputum etc. Saliva is one of the bio-fluid that has gained a lot of attraction being a non-invasive fluid that can be a diagnostic tool used for analysis of diseases in humans [1]. In the earlier period the saliva was mainly studied for its role in the oral cavity rather than in assessing the systemic diseases. With the advancements in molecular research, the role of saliva as a bio-fluid for the diagnosis of disorders and diseases has been surfaced [2].

In recent years, human saliva has emerged with lots of potential to be used as a diagnostic tool that can revolutionize the next generation diagnostics in a way that is safe, easy, inexpensive and non-invasive. This is because of the molecules such as proteins and nucleic acid that are present in saliva and can reflect the physiology of an individual [2, 3]. The challenge arises when the scientific finding of these biomolecules emerge from basic research studies. Now to translate such information to a clinical test is a bit complicated process. Fortunately, advancement in technology has resulted in methodological developments that ultimately leads to application side of such studies. Human saliva proteomics has shown to be a worthy approach to study for protein biomarkers which could identify human health and disorders. Even though much progress previously accomplished in saliva proteomics, efforts has been continuously done towards

systematizing saliva collection using different innovative devices [4] as well as pretreatment before storage so that it could become a diagnostic fluid sample.
In Pakistan there is no such proteomic study that has been conducted on saliva. The research reports from Pakistan describe the detection of Human Papillomavirus (HPV) from lesions of oral cavity through real time PCR [5] and cytokines IL6 and IL8 together with HPV 16 and 18 using enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) [6]. The proposed project aims to analyze the salivary proteins from normal healthy individuals and individuals suffering from dental caries. The protein profile was analyzed by gel electrophoresis and the concentration of immunoglobulin specifically secretary IgA (sIgA) was determined by ELISA. The results showed variations between healthy and disease samples.

Method and Material

Sample collection

The study ethical approval was taken from Institutional Ethical Committee, University of Karachi (IBC KU-94/2020). The study design was convenience (nonprobability) with suitable sample size including individuals of either sex with age range of 20-50 years with no history of pan/gutka or smoking, diabetes or any other disorders for at least three months. A dentist performed clinical examination and then saliva samples were collected from subjects by salivate in a sterile tube between 9 AM and 11 AM. Afterwards samples were spin at for 10 minutes at 10,000 g in a temperature controlled centrifuge. Aliquots of each sample were prepared and stored at -20 °C until further use. A total of 12 healthy controls and 33 dental caries patient saliva samples were collected. The data of 10 individual healthy control and 20 individual dental caries saliva samples for SDS-PAGE and zymography analysis. ELISA analysis represents all samples.

Characterization of saliva proteins

The band pattern of proteins and peptides was observed by using SDS-PAGE with 12 % gels. Samples were run under non-reducing (without β-mercaptoethanol) and denaturing conditions or reducing and denaturing conditions (with SDS, 2-mercaptoethanol/dithiothreitol and 100 °C temperature) at constant voltage of 70 volts for approximately 2 hours. Afterwards bands were visualized by coomassie blue staining as previously reported [7].

Zymography

Protease based enzyme activity of saliva samples were analyzed on 12% polyacrylamide gels copolymerized with gelatin (0.3 %) as a substrate under non reducing conditions with same procedure as of SDS-PAGE. The gels were treated twice with 10 mM Tris-HCl, containing 2.5% Triton X-100 solution pH 8.0 for 30 minutes. The gels were then incubated in 10 mM Tris-HCl containing 10 mM calcium chloride pH 8.0 for overnight incubation at 37 °C. The next day gels were stained with coomassie blue for 2 hours and destained for activity observations [7].

2D-gel electrophoresis and image analysis

For two dimensional gel electrophoresis (2D gel) was performed as previously described [4] followed by coomassie staining procedure.

ELISA analysis

To observe the difference of sIgA in the samples, Human sIgA ELISA Kit Catalog No: E-EL-H1275 96T was utilized. The supplier provided ELISA procedure was followed. Samples were run in duplicate, and read at 450 nm. The absorbance and concentration of individual samples were presented. All the concentrations were determined from the standard curve obtained from the standard solution provided with the kit. Statistical analysis was reported previously [7].

Results

The SDS-PAGE gels were run for all individual samples under non-reducing as well as in reducing conditions. Usually proteins are soluble in sample diluting buffer of SDS-PAGE procedure that allowed good resolution. It presented the separation of proteins bands in the range of 250-10 kDa for diseases samples whereas the normal healthy samples showed bands from 250-27 kDa. The bands were observed to be well resolved in reducing conditions as compared to the non-reducing in healthy as well as in dental caries samples. A consistent dark band around 62 kDa represents the amylase band in all samples.

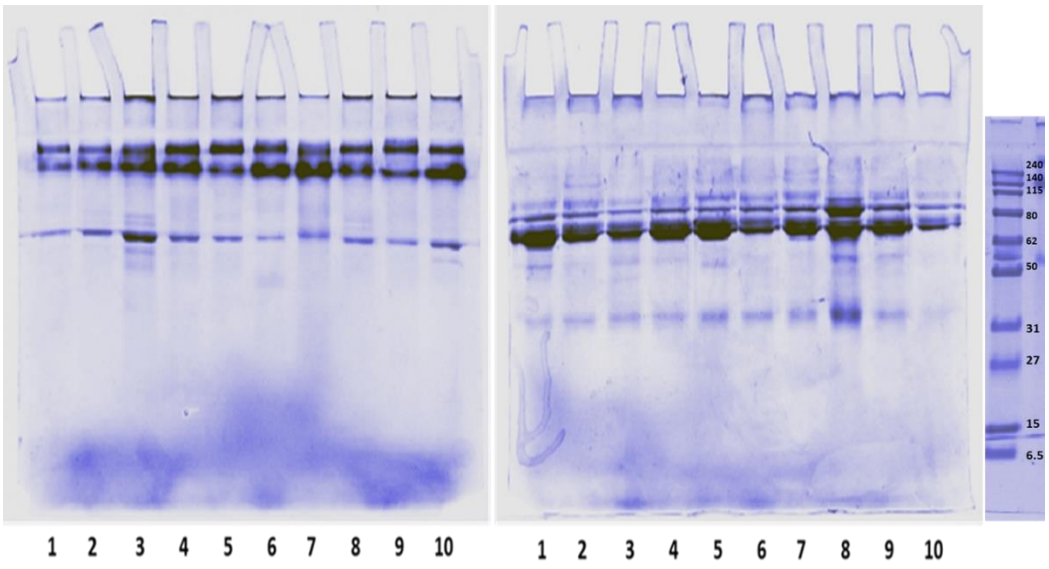
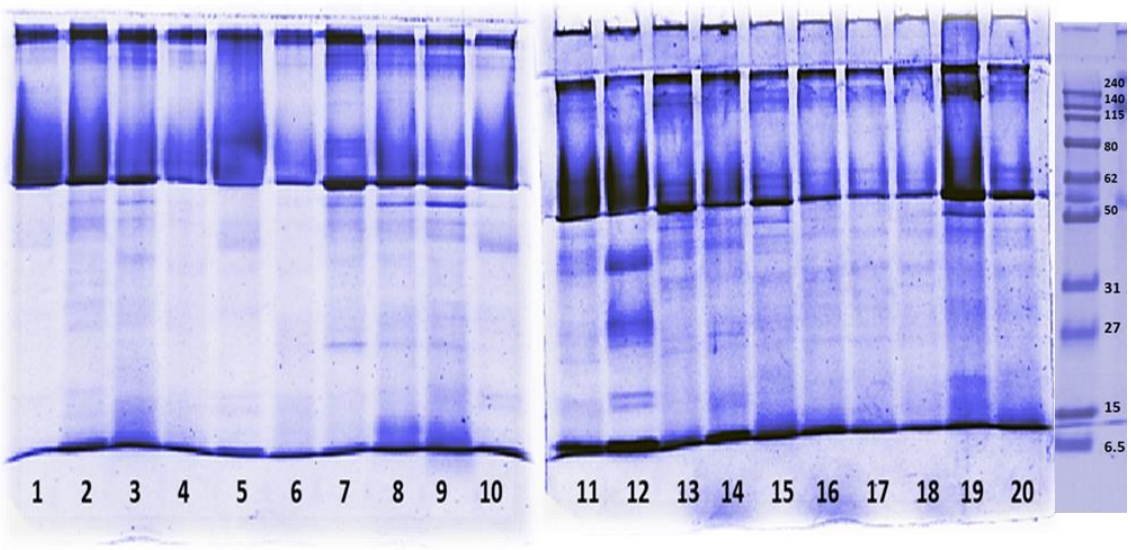


Fig. 1. SDS PAGE analysis of healthy control samples. Non reducing conditions (left) and reducing conditions (right) on 12% gel as described in methodology. The numbers at the bottom of the gel represents individual samples. Standard marker run on same percentage of gel with bands indicating respective molecular mass in kDa at the extreme right side of the figure.



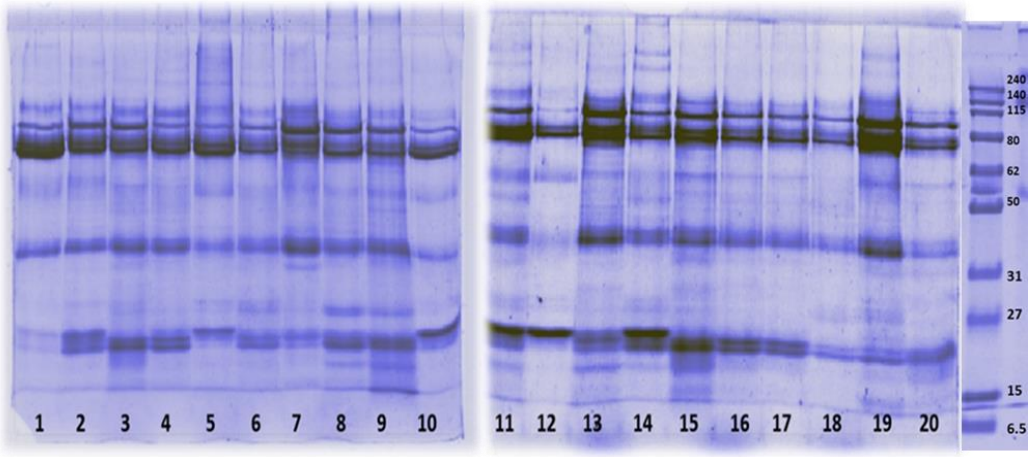
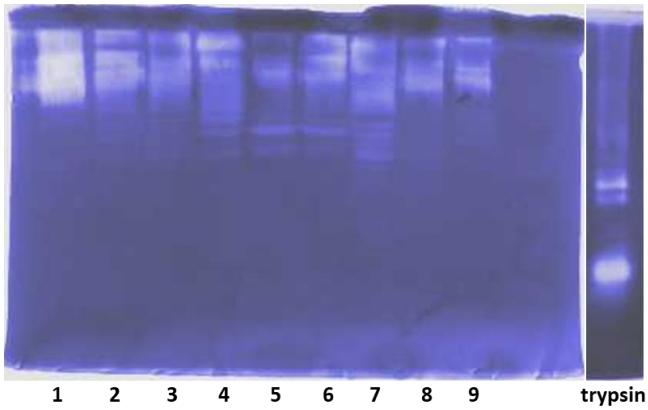


Fig. 2. SDS PAGE analysis of dental caries samples. Non reducing conditions (Upper panel of gels) and reducing conditions (lower panel of gels) on 12% gel as described in methodology. The numbers at the bottom of the gel represents individual samples. Standard marker run on same percentage of gel with bands indicating respective molecular mass in kDa at the extreme right side of the figure.

The zymography experiments performed on saliva samples from healthy controls and dental caries groups revealed clear bands in high molecular mass region of the gels. The observed bands were diffused making a streak line pattern in the respective lanes. Apparently there was a clear difference of the activity in healthy control and dental caries samples (figure 3) where there was less activity in healthy controls as compared to the dental caries saliva.



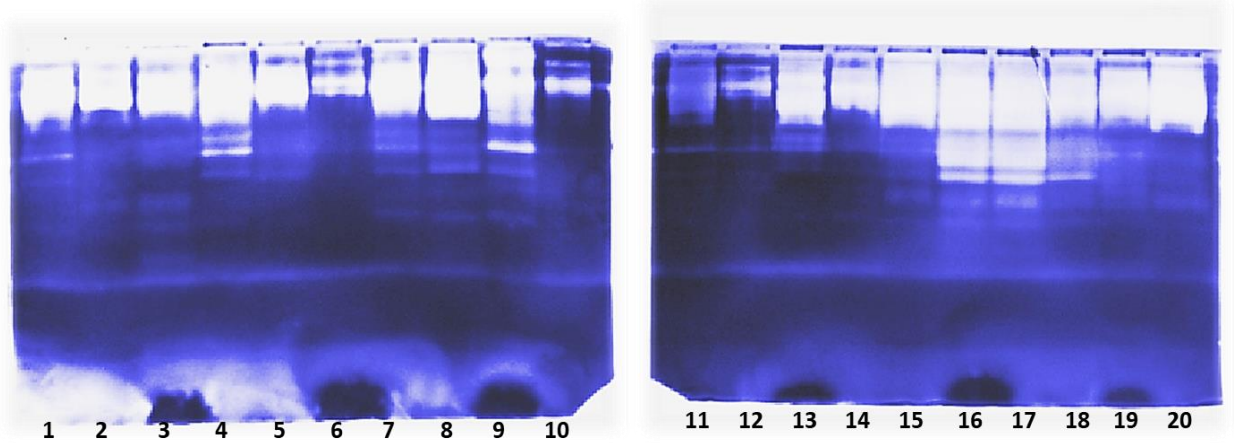


Fig. 3. Zymography of healthy control and dental caries samples (~5ug) under non reducing conditions. Upper panel represent healthy control samples and trypsin as standard (1ug). Lower panel of gels

represents dental caries on 12% gel as described in methodology. The numbers at the bottom of the gel represents individual samples.

A representative 2D gel of a healthy control saliva sample was run under conditions as mentioned in methods and material. The gel was run to have an observation regarding the low molecular mass proteins that were not visualized in simple SDS-PAGE analysis. The 15% SDS-PAGE of two dimensional gel electrophoresis exposed the presence of proteins although in low abundance in the range of 5-8 pH region as indicated with arrow in figure 4.

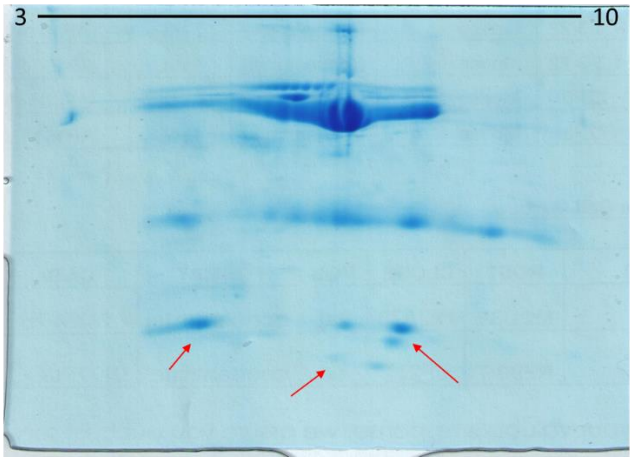


Fig. 4. A representative 2-D gel of Healthy Control Saliva Sample (sample 8). Saliva from healthy individual (80µg) was applied to an IPG strip (Bio-Rad) of pH 3-10 following equilibration, the strip was run on 15 % SDS-PAGE at constant voltage of 70 volts. Gels were visualized after coomassie staining to The line indicates the IPG strip pH gradient (3-10). Red arrows indicate the low molecular mass proteins detected on the gel.

The ELISA results showed the presence of secretory IgA antibody in all healthy control and dental caries samples. The caries samples showed high absorbance (OD) suggesting high concentrations of sIgA as compared to the controls. The highest OD was observed in dental caries sample 28 with reading of 3.661 whereas in healthy control samples it was 2.686 of sample number 9 (N9). The lowest OD observed in dental caries was 1.198 of sample 6 whereas in healthy controls lowest OD was 0.865 of sample 4 (N4).

Sample No	N1A	N1B	N2A	N2B	N 3A	N3B	N4A	N4B	N5A	N5B	N6A	N6B	N7A	N7B	N8A	N8B
Absorbance	1.424	1.336	2.624	2.357	1.824	2.032	0.865	0.934	2.046	2.09	2.292	2.342	2.166	2.334	1.838	1.315
Concentration (ng/mL)	24.83117256	23.1451	47.8224	42.7068	32.4949	36.4801	14.1211	15.4431	36.7483	37.5913	41.4615	42.4195	39.0474	42.2662	32.7631	22.7428

Sample No	N9A	N9B	N10 A	N10B	F 1	F2	1	1	2	2	4	4	6	6	8	8
Absorbance	2.376	2.686	0.887	0.848	2.327	2.378	2.369	2.633	1.794	1.764	2.428	2.257	1.245	1.198	2.157	2.578
Concentration (ng/mL)	43.07087391	49.0103	14.5426	13.7954	42.1321	43.1092	42.9368	47.9948	31.9201	31.3454	44.0672	40.7909	21.4016	20.5012	38.875	46.9411

Sample No	12	12	13	13	15	15	17	17	18	18	21	21	22	22	25	25
Absorbance	3.033	2.76	1.9	2.323	2.895	2.925	2.493	2.435	1.403	1.154	3.422	3.072	3.092	2.974	2.222	2.807
Concentration (ng/mL)	55.65856696	50.4281	33.951	42.0554	53.0146	53.5894	45.3125	44.2013	24.4288	19.6581	63.1116	56.4058	56.789	54.5282	40.1203	51.3286

Sample No	26	26	27	27	28	28	29	29	30	30	32	32	33	33	34	34
Absorbance	2.13	2.271	0.518	0.623	3.661	3.301	2.442	2.135	1.95	2.206	0.901	1.06	3.199	3.27	1.195	3.073
Concentration (ng/mL)	38.35767377	41.0591	7.4728	9.48453	67.6906	60.7933	44.3354	38.4535	34.909	39.8138	14.8108	17.8572	58.839	60.1993	20.4437	56.4249

Sample No	35	35	38	38	39	39	40	40	A	A	B	B	C	C	D	D
Absorbance	2.906	3.073	2.285	2.337	0.683	0.79	2.817	3.178	1.924	1.876	2.948	2.171	2.151	2.16	3.219	3.107
Concentration (ng/mL)	53.22532949	56.4249	41.3274	42.3237	10.6341	12.6841	51.5201	58.4367	34.4108	33.4912	54.03	39.1432	38.76	38.9325	59.2222	57.0764

Sample No	E	E	F	F	G	G	H	H
Absorbance	3.291	3.372	2.12	2.17	2.935	2.651	1.762	1.582
Concentration (ng/mL)	60.60168	62.1536	38.1661	39.1241	53.781	48.3397	31.307	27.8584

Fig. 5. ELISA readings of sIgA of healthy control (n=12) (samples donated with N, F1 and F2) and dental caries (1 to 40, donated with A to H) samples (n=33). The chart shows the absorbance (Optical density at

450 nm) and concentration of sIgA (in nano grams indicated in red) of all samples in duplicate with the exception of samples F1 and F2 which were individual.

Discussion

The saliva analysis of the two groups resulted in interesting observations. The SDS-PAGE analysis reveled that the differences observed were not in the number of the protein bands but in the size or intensity that resulted in fading of bands between the lanes of the SDS-PAGE gels. Many of the bands can be observed in figure 1 and 2 to have been present in one lane and disappearing in the next lane. The same observation was noticed in the healthy control samples with most of the bands diminishing towards the bottom of the gels. The kind of SDS-PAGE gel pattern has been reported from the saliva samples together with the consistency and physiological stability of saliva from healthy individuals [8]. A study conducted on Mexican population has shown differences in number, intensity and apparent molecular weight of the protein bands presenting the phenotypic polymorphism between individual samples [9]. It has been reported that mucins and proline rich protein (PRP) may alter with age and oral conditions. This also leads to variation in band pattern as observed in SDS-PAGE analysis. Mucins are proteins with molecular weight range from 100-200 kDa whereas the molecular weight of PRP1 for example is 30 kDa [10, 11]. Following these results, pooled samples of healthy control and dental caries were also analyzed that showed no observable differences in band pattern between the groups regardless of the increasing concentrations [7]. In different oral disease the gel electrophoresis analysis has been conducted to observe the protein bands that may be of difference between healthy and disease samples and then confirmed with other analytical techniques to conclude the findings [12]. Here the gels were run to have an overall profile of protein bands.

The zymographic analysis utilizing gelatin as a substrate showed diffused activity bands. This observation could be due to increased proteases action as well as due to close vicinity of the multiple proteases. The pattern is observable in non-reducing SDS-PAGE gels (figure 1 and 2). This proteolytic action showed the presence of highly active metalloproteinases in saliva. Trypsin enzyme was used as standard in approximately 1-2 µg concentration which is a 25 kDa serine protease. These activity bands may include bands of ~140, ~80, ~62, ~50 and ~40 kDa molecular weight [7]. The genetic polymorphism in matrix metalloprotease has been studied and it has been suggested that MMP13 gene polymorphism may have a relation with dental caries [13]. It is a 60 kDa protein investigated in different oral disorders [14]

The 2D gel showed majority of spots being concentrated to high molecular mass area on the gel. This effect could be due to major proteins such as amylase and immunoglobulins. These protein can interact with other proteins in saliva to form an interactome. For example it has been described that at least 66 diverse proteins can interact with amylase. This suggest an additional function of this enzyme for protection and transport of its partner proteins [15].

Two dimensional gel utilizing 4-7 pH IPG strip revealed that most of the spots identified were that of amylase and albumin [16]. In another study these major protein components of saliva were (including albumin and amylase) depleted by acetone treatment and then depleted sample was analysed through 2D gel for periodontal patients [17] This kind of approach may reveal much resolved spots of protein for identification of differences between the healthy control and disease groups.

Different antibodies in the body fluid are responsible for the immune response. Secretory immunoglobulin A establishes the key immune response in saliva and play a significant part in the homeostasis of the oral health. The concentration of such antibodies vary through out the life of an individual with incline and decline of the age and gender [18].

The results of sIgA antibody up regulation are in agreement with the earlier reported data [19]. The functioning of immune response related to the decay of tissues such as tooth or the surrounding gums would result in increase in the normal concentration of antibodies. The

response is an attempt of the body to control the condition and stop the spread of the agent causing the disorder. There are contradictory views on the nature of sIgA action. For example, as sIgA binding with cariogenic microbiota in caries-susceptible patients the concentration of sIgA in saliva will go down. The other argument is that such binding will trigger the immune response further which would lead to an increase in sIgA [20]. It has also been reported that the correlation of salivary sIgA in dental caries patients with respect to the occurrence of cavity on the tooth surface (lateral teeth surface cavity) showed high levels in healthy controls as compared to dental caries patients. The study also included the presence of interferons and reported the findings in combination to sIgA [21]. Our results supports the availability of free sIgA in the saliva [7].

Conclusion

Our results demonstrated that there is a difference in saliva of healthy control and dental caries patients. The presence of differentially expressed bands in SDS-PAGE analysis represents the phenotypic polymorphism among the individual samples. Over all there is an increase in proteolytic activity and immunogenic response in dental caries patients representing the infection or disease condition.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. The study on humans was conducted in accordance with the ethical rules of the Helsinki Declaration and Good Clinical Practice.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

None.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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